University of Alberta

Phytoremediation of Soils Contaminated with Phenanthrene

by

Kristi Michelle McLachlan

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Master of Science*

in

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ABSTRACT

Phytoremediation, the ability of plants to accelerate decontamination in soils contaminated with petroleum hydrocarbons, is a potentially cost-effective means of remediating contaminated sites. The primary mechanism of this attenuation is believed to be the enrichment of microorganisms that break down the contaminant along the root surface. Wheat was selected as the model plant based on preliminary studies that showed a high root surface area for microbial colonization, in addition to being moderately tolerant to phenanthrene, a polycyclic aromatic hydrocarbon. Enumeration of bacteria in the rhizosphere (the soil adhering to the root surface) showed 5-15 times more phenanthrene-degrading bacteria than in unplanted soil at phenanthrene concentrations between 200-1000 mg kg^{-1}. Experiments tracking radiolabelled phenanthrene in a closed flow-through plant growth chamber demonstrated the planted treatments to break down 16-18% more than unplanted treatments. These results confirm that planted treatments accelerate the decontamination process, and provide information on how to optimize future phytoremediation programs.

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CHAPTER 1. GENERAL INTRODUCTION

1.1 SOIL CONTAMINATION

Terrestrial soils contaminated with petroleum compounds present a major challenge to Canada's oil and gas industry. These contaminants represent the most widespread soil contaminants in Canada (Canada Wide Standards for Petroleum Hydrocarbons in Soil (CWS) 2001), however little information is available concerning their movement within terrestrial ecosystems. The fate of many petroleum compounds is still uncertain, so it is important that contaminated sites are remediated due to potential toxic effects of these contaminants. Potential dangers of petroleum compounds include fire/explosion hazard, human and ecological toxicity, movement through soil and the food chain, foul odour, and impairment of soil processes such as water retention and nutrient cycling (CWS 2001; McLachlan 1996). This contamination is important to vegetation, as Chaineau *et al.* (1996) showed that hydrocarbons significantly reduce plant growth, seed germination and vegetative development in soils artificially contaminated with fuel oils. Similarly, Hou *et al.* (1999) noted that seed germination of perennial ryegrass was hindered at relatively low concentrations of diesel contaminated soil. Phytotoxic effects were also observed in corn and soybean by Bossert and Bartha (1985), who demonstrated geotropic disruption of root and shoot tips by fuel oils (roots grew horizontally and/or upward while shoots and cotyledons grew sideways or downward).

In Canada and the United States, management options for contaminated sites are left up to the organization responsible for site clean up, fuelling the search for more cost-effective methods. A comparison between traditional *ex situ* (off-site) and *in situ* (on-site) methods shows that costs decrease dramatically with the use of biological methods. For example, Chappell (1998) and Jipson (1996) projected the costs of remediating a site contaminated by petroleum hydrocarbons (size not disclosed) using *in situ* remediation techniques to be $70,000 United States Dollars (USD) compared to $850,000 USD for currently used technologies. Other estimates include $1,000,000 USD per hectare to remediate to a depth of half a metre for traditional methods and $2,500-$15,000 USD per hectare, including analysis and consulting, for *in situ* biological techniques (Cunningham *et al.* 1996). Given these dramatic cost differentials, it is clear why bioremediation is so attractive to Canada's oil and gas industry.

1.2 Soil Decontamination Strategies

Current *ex situ* clean-up methods require the transportation of a contaminated soil to an off-site facility where various methods may be employed, including incineration or bioslurry treatment. These methods are adequate but costly, and the potential of a less costly and labour intensive attenuation procedure is attractive. *In situ* biological attenuation (bioremediation) of contaminants can occur as a result of microbial or plant processes. Several methods to achieve bioremediation have been investigated.

1.2.1 *In situ* Bioremediation

In this paper, "bioremediation" refers to the breakdown of a contaminant by microbial processes, although in industrial applications, the term is also used to encompass all forms of biological or natural *in situ* remediation. Numerous laboratories have demonstrated mineralization of many petroleum compounds by microorganisms. Aromatic hydrocarbons are degraded primarily by bacterial and fungal enzymes through cleavage of the aromatic ring structure, facilitating further breakdown in the soil environment. Polycyclic aromatic hydrocarbons (PAHs), major components of some petroleum mixtures, are typically degraded by the incorporation of oxygen onto the ring structure with microbial oxygenase, generating a dihydrodiol (Qiu 1997). Aitken *et al.* (1998) tested ten strains of bacteria from seven different contaminated soils and found that all could mineralize a broad range of PAHs, including phenanthrene, benz[a]anthracene, chrysene and benzo[a]pyrene.

A variety of bacterial strains are capable of completely mineralizing petroleum hydrocarbons, ultimately resulting in the production of carbon dioxide. Contaminant degradation by microorganisms has been repeatedly demonstrated in laboratory settings (crude oil, Salanitro *et al.* 1997; fuel hydrocarbons, Friello *et al.* 2001; diesel fuel, Gallego *et al.* 2001; phenanthrene, Aitken *et al.* 1998; Anderson *et al.* 2001; MacGillivray and Shiaris 1994). Gallego *et al.* (2001) determined that *Acinetobacter* spp. were responsible for much of the degradation observed in diesel-contaminated soils

within a bioreactor. Although laboratory culture organisms proliferate and degrade petroleum hydrocarbons rapidly under controlled conditions, they are typically less successful under field conditions.

Initially, the primary focus of bioremediation work was the development of genetically engineered microorganisms (GEMs) able to degrade a target xenobiotic for introduction into a contaminated environment. This has been successful in the laboratory. For example, Friello *et al.* (2001) successfully used mutants of *Sphingomonas paucimobilis* to degrade PAHs. However, in cases where GEMs have been introduced to the field, indigenous microbes with a history of exposure to contamination were quickly able to outcompete their laboratory counterparts. In other cases, the introduced organisms simply fail to colonize (Pritchard 1992). Potential reasons for this failure include problems in adhering to an alien matrix, the impact of predation, and difficulty acclimating to limited nutrient conditions (Alexander 1999; Gallego *et al.* 2001). In the past decade, research has begun to shift toward maximizing the ability of indigenous microorganisms to utilize anthropogenic compounds as growth substances.

1.2.2 Biostimulation

Biostimulation involves the addition of soil amendments such as environmentally benign structural analogues (Alvarez *et al.* 1998), electron donors or acceptors, and/or fertilizers to stimulate degradation by indigenous microorganisms. Alvarez *et al.* (1998)

exposed a bacterial consortium from a benzene/toluene/xylene (BTX) contaminated site to benzoate, an intermediate in the toluene degradation pathway, to induce related enzymes in the degradation of toluene and *m*- and *p*-xylenes. Benzoate shortened the acclimation period and enhanced short-term degradation potential of the consortium. Lin and Mendelssohn (1998) observed higher microbial numbers and metabolic activities in fertilized soils compared to unfertilized treatments. The authors also observed an increased crude oil degradation rate in fertilized soils. Biostimulation of microbes by hand-spreading fertilizer is currently being employed at diesel-contaminated sites in Alberta (Nancy McIntyre, Environmental Group, Telus Communications, Inc., personal communication).

1.2.3 Phytoremediation

Phytoremediation, the use of green plants to expedite degradation, has also received widespread attention. Plants can enhance degradation via several possible mechanisms: 1) plant assisted rhizosphere mineralization, 2) uptake and subsequent degradation or sequestration in plant tissues, or 3) plant-enhanced bioremediation. In addition, plants provide a number of beneficial aspects to a clean up site. These include containment of contaminated soil as plant cover reduces wind and water erosion (Joner *et al.* 2001), improved soil structure and aeration, and increased surface area for promoting microbial colonization.

Enzymatic transformation

Extracellular or membrane-bound plant enzymes can be responsible for the transformation of contaminants (Dietz and Schnoor 2001). Plant enzymes such as dehalogenases, nitroreductases, nitrilases, oxidoreductases and laccases have been discovered in soil and sediments at a distance from their origin (Cunningham *et al.* 1996; Adler *et al.* 1994). The mode of action of most oxidoreductases (such as peroxidases) is hypothesized to be the polymerization of the pollutant either onto the root surface or into soil organic matter, thus making it unavailable for any other biotic or chemical processes (Cunningham *et al.* 1996). These enzymes have been found on external root surfaces of onion, wheat, cress, tomato and water hyacinth (Cunningham *et al.* 1996).

Fungal symbionts, parasites and saprophytes sharing the soil environment with plants produce a wide variety of enzymes that may also be involved in degradation (Bollag *et al.* 1994). Bressler *et al.* (2000) showed that fungal laccase serves to break the aromatic ring structures of certain aromatic compounds, such as carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene. It is possible that some rhizosphere plant laccases may aid in the degradation of PAHs.

Uptake and phytotransformation

Natural attenuation by plants may occur via uptake and sequestration or degradation within tissues of the plant. Due to the potential carcinogenic properties of certain petroleum compounds, there is concern that a pollutant may be translocated

through the plant by the xylem (Simonich and Hites 1995), and pose a potential hazard through bioconcentration within the food chain. Models predicting plant uptake of hydrocarbons in soil-plant systems have been developed based on lipid solubility (lipophilicity) of a compound (Briggs *et al.* 1982), root growth distribution (Chang and Corapcioglu 1998) and microbial activity in the root zone (Corapcioglu 1992). These were designed to predict the fate of agricultural pesticides, however several subsequent laboratory experiments showed that these models may not accurately predict the fate of organic contaminants in soil (Orchard *et al.* 2000a,b; Burken and Schnoor 1997) and imply a lower potential for use in modelling contaminant clean-up than previous theory suggests. With respect to petroleum hydrocarbons, Chaineau *et al.* (1997) suggested that it is unlikely that PAHs enter the plant, but biogenic metabolites may.

The potential of an organic compound to enter a plant from the soil depends on a number of physical and chemical properties of the compound. Water solubility, Henry's Law Constant, and the octanol-water partition coefficient (K_{ow}) are all important factors in determining whether the compound can be absorbed into the root and transported through the plant (Simonich and Hites 1995). Polycyclic aromatic hydrocarbons and other compounds with a log K_{ow} of greater than 3-4 are not expected to enter the xylem stream and be transported to the above-ground plant portion. They are more likely to remain partitioned in the epidermis of the root due to their lipophilic properties (Simonich and Hites 1995; Dietz and Schnoor 2001). To enter the xylem stream via a symplastic pathway, a moderately hydrophobic compound must cross at least two

membranes. The first passage is through the root cell membrane. Subsequently, the compound is passed from protoplast to protoplast through plasmodesmata (symplastic transport). If the compound can flow through the cortex and across the endodermis into the stele in this manner, it must then pass through a stelar cell membrane into the apoplast to enter the xylem stream. Hydrophilic compounds can quickly move through the intercellular space (apoplastically) along with the mass flow of water until reaching the waxy Casparian Strip barrier in endodermal cell walls. To pass through to the stele, solutes must enter endodermal protoplasts and pass through the barrier via the symplast. Regardless of the transport pathway through the cortex, once the Casparian Strip has been crossed, compounds must enter the apoplast to enter the xylem vessels. If hydrophilic solutes are able to pass into endodermal protoplasts, they are much more likely to reach above-ground portions of plant tissues. A compound with a high K_{ow} rarely reaches the xylem stream due to an affinity for lipophilic tissue constituents such as cell membranes and the Casparian Strip itself.

Most petroleum hydrocarbons are highly lipophilic and not likely to flow through the symplast or apoplast. One important constituent of petroleum is the PAH fraction. One possible component of a petroleum mixture, naphthalene, has a two-ringed aromatic structure that is volatile, making it an unlikely compound for plant uptake due to its low residence time in soils (Aprill and Sims 1990). It is also the PAH with the lowest log K_{ow} (3.29) of those typically found in soils contaminated with petroleum hydrocarbons. The PAH with the next highest log K_{ow} (4.2-4.6) is phenanthrene, a three-ringed structure

found in most fuel and diesel oils. The moderate lipophilicity of this compound makes it an interesting target for fate and transport studies.

The Transpiration Stream Concentration Factor (TSCF) is a measure of the movement of a compound into the plant. The TSCF is a ratio of the concentration of the chemical in the xylem sap to the concentration in the root zone solution. The TSCF is also used in a model developed to predict the behaviour of a compound in a root matrix-plant system. Theoretically, a TSCF of 1.0 indicates unrestricted, passive uptake; less than 1 indicates that the plant is excluding the contaminant; greater than 1 indicates active uptake by the plant (Briggs *et al.* 1982; Orchard *et al.* 2000b; Schnoor *et al.* 1995). Briggs *et al.* (1982) established the following empirical relationship to calculate the TSCF using *o*-methylcarbamoyloximes and substituted phenylureas;

$$TSCF = 0.784 \exp \{- [(\log K_{ow} - 1.78)^2/2.44]\} \quad (1)$$

Burken and Schnoor (1998) tested this relationship using trichloroethylene (TCE). The revised relationship was defined as;

$$TSCF = 0.784 \exp \{- [(\log K_{ow} - 2.50)^2/2.58]\} \quad (2)$$

The relationship between the TSCF and log K_{ow} has been widely used to predict chemical movements in plants. Orchard (2000b) tested the relationship using ^{14}C-TCE and found TSCF values 4-10 times lower than the Burken and Schnoor's (1998) calculated TSCF suggested. McFarlane *et al.* (1987) suggested log K_{ow} may not be useful as a predictive tool for uptake of nonpesticide chemicals. This conclusion arose due to a lack of similarity in measured TSCF values in soybean when exposed to soil bromacil, phenol or nitrobenzene, all compounds with similar log K_{ow} values. Although it is generally accepted that lipophilic contaminants are not likely to enter the plant through the roots, there have been some exceptions. Hulster *et al.* (1994) found that zucchini and pumpkins were capable of accumulating unlabelled polychlorinated dibenzo-p-dioxins and dibenzofurans as the parent compounds (log K_{ow} >6; predicted TSCF = 5.91).

In addition to uptake and sequestration, a few degradation pathways have been noted in plant cell cultures. For example, degradation of benzo[a]pyrene to oxygenated derivatives, amino acids and carbon dioxide has been demonstrated in cell cultures of *Chenopdium rubrum* (Harms *et al.* 1977). These findings contradict expected results, since benzo[a]pyrene is a PAH with a log K_{ow} of 6.04, and as such is not expected to enter plant tissues based on the TSCF value of 6.1 (calculated using equation (1)).

Phytovolatilization may also occur after contaminant uptake. Once the contaminant has been translocated through the xylem of the plant, the chemical or a

metabolite thereof may be volatilized (Schnoor 1997; Salanitro *et al.* 1997). This scenario may not always be ideal, however the process does release the contaminant into the atmosphere, diluting and dispersing the contaminant.

The abundance of plant uptake theories supported by limited validation has led some researchers to design plant growth systems in which the path of an organic compound can be traced. Orchard *et al.* (2000a) introduced a novel method to trace contaminants through a hydroponic matrix-plant system by separately trapping organic compounds volatilized by shoot and root portions of the plant. This method is well suited to trace the fate of petroleum hydrocarbons through a soil-plant system. The foliage of the plant is separated from the root system, and volatile products and carbon dioxide are trapped in organic solvents. With ^{14}C-radiolabelled compounds trapped where they are degraded, their location and the extent of mineralization can be determined.

Plant-Assisted Bioremediation – The Rhizosphere

The rhizosphere is the region surrounding the plant root containing soil particles, microflora and fauna, plant exudates, and dead root cells. The rhizosphere is the soil at the soil-root interface where dissolved oxygen and carbon dioxide concentrations, oxidation-reduction potentials, pH, and moisture content differ from bulk soil (Foster 1981; Grayston *et al.* 1996). Microbial growth in soils is generally carbon limited (Grayston *et al.* 1996), and plant roots provide carbon in the form of sugars, vitamins, and amino acids. Up to 40% of the net carbon fixed during photosynthesis can be

released into the rhizosphere (Yoshitomi and Shann 2001; Lynch and Whipps 1990). Sugars and amino acids are the first and second most abundant exudate components, respectively (Juma and McGill 1986; Jaeger *et al.* 1999). Other important components of the rhizosphere are plant mucilages, lubricants excreted by the roots as they penetrate the soil (Cunningham *et al.* 1996), and vitamins and cofactors necessary for growth of certain microorganisms (Rovira and Harris 1961; Yoshitomi and Shann 2001). Root cell turnover is also a large contributor to available soil carbon, as root cap cells may be lost to the soil at a rate of 10 000 cells plant^{-1} day^{-1} (Campbell 1985; Cunningham *et al.* 1996). This plant-mediated increase in assimilable carbon increases microbial growth in planted soils. Another major component of the rhizosphere is mucigel. Rhizosphere microorganisms secrete polysaccharides in the form of gels, slimes and capsule materials (Webley *et al.* 1952). These become inextricably mixed with plant exudates and mucilages to form mucigel (Foster 1981), in which bacteria can proliferate. The microenvironment surrounding the roots is not necessarily controlled solely by root carbon releases. The addition of microorganisms to the root-soil interface can increase nutrient release by roots (Lynch and Whipps 1990; Grayston *et al.* 1996). Prikryl and Vancura (1980) have also demonstrated that the presence of *Pseudomonas putida* and *Azospirillum* spp. increases carbon release by the plant.

Microbial populations and activity in the rhizosphere can be as much as 100 to 1000-fold higher than in bulk soil (Paul and Clark 1989; Reilley *et al.* 1996; Crowley *et al.* 1997). This increase is known as the rhizosphere effect. In exchange for carbon in the

form of exudates and cell materials, microorganisms help solubilize insoluble nutrients, recycle organically-bound nutritive elements for the plant, decrease the vulnerability of roots to pathogens, synthesize and release growth factors, and decompose dead plant matter (Cunningham *et al.* 1996; Walton and Anderson 1990). A few studies have disputed the extent of the rhizosphere effect. Boyle and Shann (1995) suggest that the number of organisms may in fact be similar between rhizosphere and nonrhizosphere soils, and Crowley *et al.* (1997) caution that high numbers do not necessarily translate to high metabolic activity.

Given the attractiveness of the plant uptake theory, the presence of metabolic plant enzymes, and the knowledge that microorganisms can adapt to contamination, it makes sense to investigate these mechanisms together to maximize decontamination. Few studies have focussed on the relationship between plant roots and microbes for remediation purposes, even though the rhizosphere effect is recognized by many researchers (Crowley *et al.* 1997; Cunningham *et al.* 1996; Walton and Anderson 1990). If the theory of increased rhizosphere microbial population is correct, plant species with extensive and fine root systems should have the greatest potential for enhancing bioremediation. These root systems would have greater root-soil surface contact, thus greater microbial growth surface area than a coarse taproot system (Cunningham *et al.* 1996).

Some investigators have begun to explore the relationship between increased bacterial numbers in the rhizosphere and the potential for increased contaminant degradation. Microbes found in the rhizosphere of some plants are capable of using petroleum hydrocarbons as a carbon source (Salanitro *et al.* 1997) and sites could be planted to improve the remediation capacity of a site. Walton and Anderson (1990) demonstrated that microbial degradation of TCE occurred seven times faster in soil containing plant material (rhizosphere) than in non-vegetated soil. More recently, Lin and Mendelssohn (1998) found that the application of fertilizer enhanced the capacity of plant-soil systems to degrade petroleum hydrocarbons, and suggested that organic substances released by roots improved soil conditions for microbes to further degrade organics in the soil.

There are many hypotheses regarding the mechanisms that might account for observed increases in the rate of degradation of petroleum compounds in soil. The main explanation is increased bacterial density in the rhizosphere. It has also been shown that the metabolic activity of microorganisms near the plant root is greater than that of microorganisms in bulk soil (Cunningham *et al.* 1996). Plants may also aid in the degradation process by creating microenvironments conducive to plasmid transfer and increased genetic exchange. Cometabolism may also be enhanced via rhizodeposition of cosubstrates that induce the enzymes for catabolism of soil contaminants (Crowley *et al.* 1997). Plant-derived compounds may also act as structural analogs to contaminants and

alter the community by selecting for degrading microbial populations (Sandmann and Loos 1984; Boyle and Shann 1995).

Although current studies imply the importance of plant-microbe interactions in the rhizosphere, no study to date has quantitatively defined these interactions with respect to degradation of petroleum hydrocarbons while soil is under the influence of a live, growing root. Miya and Firestone (2001) showed that root exudates and root debris-amended soil improved phenanthrene degradative capacity of soils, however, these experiments were conducted in the absence of actively growing plants. In contrast, Fang *et al.* (2001) found there was no difference in phenanthrene degradation between planted and unplanted soils. In this case, however, "planted" referred to soils that had been removed from a pot containing a plant, and not the rhizosphere of the experimental plants.

The rhizosphere-assisted phytoremediation hypothesis is widely accepted, yet studies to date have been primarily empirical or inconclusive. Research in phytoremediation currently focuses on organic compounds other than petroleum hydrocarbons, such as TCE (Orchard *et al.* 2000b), trinitrotoluene (TNT; Hughes *et al.* 1997), and pesticides such as atrazine (Burken and Schnoor 1997). These studies have indicated that plants cannot be used alone for efficient uptake of these contaminants. Other phytoremediation studies have dealt with soil outside the influence of a growing plant (Walton and Anderson 1990; Wetzel *et al.* 1997; Miya and Firestone 2001), have

been lacking in contaminant recovery (Chaineau *et al.* 1997; Reilley *et al.* 1996), have demonstrated inadequate mass balance (April and Sims 1990), or have been short-term studies not applicable to actual field situations (Burken and Schnoor 1997).

Quantitative data on the remediation of petroleum hydrocarbons are inconsistent and isolated, focusing primarily on plants or microbes separately. Aprill and Sims (1990) found that PAH disappearance was greater in vegetated soil, however, their experimental design included mixing a combination of PAHs with manure and failed to take into account sorption of experimental compounds to organic matter. Wetzel *et al.* (1997) found no rhizosphere effect (degradation rate improvement in soil exposed to root influence) when soil was exposed to pyrene, a four-ringed PAH, or anthracene, a three-ringed PAH. Once again, this experiment was conducted outside the influence of an actively growing root and used soil only previously exposed to plant growth. Unexpectedly, Ferro *et al.* (1994) did not find any significant difference between planted and unplanted controls in the mineralization of phenanthrene. However, this experiment did not have any sterile controls to isolate the effect of microorganisms in the system.

1.3 OBJECTIVES

The primary objective of this research was to determine whether the unique characteristics of rhizosphere soil increase the rate of degradation of petroleum hydrocarbons in contaminated soil. The specific objectives of the research include:

1. To develop of a model system (soil, plant, microorganism) for laboratory investigation of biological degradation of petroleum hydrocarbons.
2. To assess of the extent of petroleum hydrocarbon degradation in plant tissue, rhizosphere soil and sterile contaminated soil, contaminated soil with indigenous microflora, and contaminated soil inoculated with a community of petroleum hydrocarbon-degrading microbes.
3. To determine of the fate of a model radiolabelled contaminant in the model soil-plant-microorganism system.

These objectives were achieved by developing a model soil-plant-microorganism system and using this system to monitor the degradation of ^{14}C-labelled phenanthrene by plants and microbes in soil. Root and shoot compartments were separated and a modified version of the plant growth system introduced by Orchard *et al.* (2000a) was used. Conclusions obtained from this research will aid in the evaluation of phytoremediation as a potentially cost-effective alternative method of soil decontamination, isolate the optimal treatment combination for hydrocarbon degradation, and provide a basis for selection criteria and application guidelines for phytoremediation purposes.

1.4 Working Hypothesis

The working hypothesis of this research was that the primary mechanism of petroleum hydrocarbon degradation is microbial breakdown; thus the rates of degradation

should be higher in treatments containing indigenous microorganisms from a contaminated site compared to soil without bacteria. It was expected that the plant would exert a small (but significant) effect on the rates of hydrocarbon degradation. Due to the high log K_{ow} of certain petroleum hydrocarbons it was also expected that the model plant would take up little to no contaminant.

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CHAPTER 2. SELECTION OF A MODEL CONTAMINANT, PLANT AND MICROBIAL CONSORTIUM FOR PHYTOREMEDIATION STUDIES.

2.1 INTRODUCTION

Phytoremediation has received widespread attention over the past decade as a potential soil decontamination strategy. In spite of the enthusiasm expressed over the use of this strategy, success with this type of treatment will increase when the intricate relationships between plants, soils and microorganisms are understood. A closed flow-through system geared toward separating the root and shoot portions of a plant-soil system would be ideal to elucidate some of these key inter-relationships. To simplify experiments in this flow-through system, it is important to select a model plant and bacterial consortium with which to conduct fate and transport studies.

2.1.1 The Model Contaminant

Phenanthrene was selected as a model contaminant for fate studies. The main criteria for selecting phenanthrene included low volatility (vapour pressure of 0.45 Pa), presence in petroleum-contaminated soils, biodegradability (persistent yet can break down over the course of this research), bioavailability, and the potential for toxic effects. Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) found in petroleum-contaminated soils. Phenanthrene is also moderately persistent, has a log octanol-water

partition coefficient (log K_{ow}) of 4.36, and is potentially toxic and bioavailable. Phenanthrene has also been used as a model contaminant in studies concerned with *in vitro* microbial degradation (Kiyohara *et al.* 1982), soil degradation rates (Ferro *et al.* 1994), and rhizosphere microbial studies (Fang *et al.* 2001).

2.1.2 The Model Plant - Toxicity of Phenanthrene

It is generally accepted that microbial degradation is the primary breakdown mechanism in soils contaminated with PAHs (Cunningham *et al.*1996; Crowley *et al.* 1997; Siciliano and Germida 1998). A model plant in a phytoremediation study would thus be one that could support high numbers of heterotrophic and phenanthrene-degrading microbes along the root surface. Researchers have suggested that fibrous root systems provide a higher surface area for microbial colonization compared to tap root systems (Cunningham *et al.* 1996; Alexander 1999). However, the tap root systems of certain legumes could also be beneficial due to the presence of a more diverse array of heterotrophic bacteria, including nitrogen-fixing rhizobia.

A second important parameter in the selection of a model plant is the toxicity of phenanthrene. Few studies have investigated the effects of PAHs on whole-plant growth in terrestrial systems. Henner *et al.* (1999) concluded that there was little to no inhibition of plant germination or growth in soils contaminated with three-five ringed PAHs. In soils contaminated with 294 g kg^{-1} soil oily sludge, Bossert and Bartha (1985) noted

severe inhibition of germination in soybean and severe inhibition of growth in corn. They hypothesized that this was a likely result of the structural similarities between plant growth hormones and compounds in the oily sludge.

A plant that could tolerate levels of phenanthrene found in specific contaminated soils, in addition to being able to germinate in collected field soil, would be a good candidate for a model plant. The selected plant should also be able to sustain high numbers of bacteria along the root surface.

2.1.3 The Model Consortium – Microbial Populations in the Rhizosphere

Bacteria in soil can degrade phenanthrene via two mechanisms. It can either be used directly for metabolic processes or it may be cometabolized. Cometabolism involves transformation by microbes that do not use the compound for growth or metabolic processes. A model microbial consortium will include bacteria that can undergo both of these mechanisms.

Boyle and Shann (1995) suggested that a qualitative, not quantitative, difference in bacterial populations exists in rhizosphere soils. They showed that microbial activity (measured as mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid) was highest in monocot rhizosphere soils, followed by dicot rhizosphere soils, followed by non-rhizosphere soils. Their study used soils harvested

from the field that were previously exposed to the influence of plant roots, rather than direct soil-root contact for the duration of the experiment. This ranking according to plant type was contradicted by Nichols *et al.* (1997), who found the alfalfa rhizosphere supported higher numbers of heterotrophic and organic chemical-degrading microorganisms than the bluegrass rhizosphere. The difference between this experiment and that of Boyle and Shann (1995) is that the bacteria were counted from soil collected from the actively-growing rhizosphere of both species.

Among certain grass species grown in two different soil types, Grayston *et al.* (1998) showed the wheat rhizosphere contained higher heterotrophic bacterial populations than the rhizosphere of ryegrass or bentgrass. However, with respect to phenanthrene-degrading microorganisms, Fang *et al.* (2001) found no difference between rhizosphere and bulk soils associated with five grass species. The above findings seem to contradict one another, depending on the methods and compounds used. No clear pattern has emerged to determine which plants would be ideal for model studies.

An important consideration in phytoremediation studies is whether the root is selectively enriching indigenous bacteria capable of degradation, or whether the contaminated environment is exerting a selection pressure that favours phenanthrene-degrading bacteria. To date, bacteria used to inoculate soils in the laboratory have been unsuccessful in colonizing the soil. Indigenous bacteria already accustomed to soil

conditions outcompete inoculated organisms (Cunningham *et al.* 1996; Pritchard 1992). This suggests indigenous bacteria are being selectively enriched.

For the planned flow-through experiments (outlined in Chapter 3), the ideal bacterial consortium (isolated from a contaminated site) was considered to be one that could degrade phenanthrene at a high rate. It was important to find bacteria that had successfully established in a contaminated soil, rather than a bacterial consortium developed *in vitro*. This should improve the chances of the consortium successfully colonizing a plant-soil system. This model consortium would be enriched and inoculated into contaminated soils to determine whether laboratory-grown microorganisms can compete with their indigenous counterparts in artificially-contaminated soils.

2.1.4 Study Objectives

The overall objective for this phase of the study was to select a model plant and isolate a bacterial consortium for use in phytoremediation studies. Wheat (*Triticum aestivum*), canola (*Brassica napus*), and alfalfa (*Medicago sativa*) were screened for a variety of responses when planted in soil contaminated with phenanthrene. Important selection parameters were: low variability; shorter length of experiment; previous uses (documented in the literature); toxicity of phenanthrene to the plant in soils artificially contaminated with phenanthrene; root colonization by phenanthrene-degrading microorganisms; germination in contaminated soils; and root surface area.

2.2 MATERIALS AND METHODS

2.2.1 Plant Growth and Toxicity Studies

Wheat germination and growth

Wheat (*Triticum aestivum*) was selected for dose response tests based on availability within the laboratory. Sterility tests of two available cultivars, Maringa and Katepwa, were conducted on Murashige and Skoog medium. Maringa was able to germinate in sterile conditions and was chosen for all tests. Seeds were surface sterilized in 20% bleach for 20 min under constant agitation (stir bar and plate) and rinsed for approximately 2 min with de-ionized water (>18MΩ; Milli-Q; Millipore; Billerica, USA). Seeds were germinated overnight in an aerated solution of 12.5 mg L^{-1} of Vitavax (Gustavson, USA). Germinated seeds were rinsed and placed in a moist paper towel for 48 h. Seeds with three roots of approximately the same size were planted in a borosilicate glass tube (24 X 4 cm) filled with a 50/50 greenhouse soil/silica sand mixture. At the bottom of each tube, approximately 10 mL of drainage stones were capped with a Kim-Kap™ polypropylene test tube closure (Fisher Scientific Co.; Nepean, Canada). Each tube received 10 mL of water before the seedling was planted, followed by 5 mL after planting. Prior to the second watering, approximately 3 mL of extra soil/sand mixture was placed on top of the seedling. The glass tubes were wrapped in opaque black plastic and placed in a growth chamber (195 µmol m^{-2} s^{-1}) with a 16-h photoperiod at 21°C. Plants were watered with 10 mL of water every other day for the

first 21 d, after which plants received 15 mL every other day for the remaining duration of 35 d experiments. The water regime was altered as necessary to ensure all soil in the root zone was wetted during the watering process.

Alfalfa germination and growth

Alfalfa (*Medicago sativa* cv. Beaver) was selected based on availability within the laboratory. Seeds were surface sterilized in 20% bleach for 20 min under constant agitation and rinsed for approximately 2 min with de-ionized water. Seeds were placed in a moist paper towel for 48 h. Seeds of approximately the same size were planted in a borosilicate glass tube as described in the wheat germination and growth section. Plants were watered with 15 mL of water every third day for the duration of the experiment (21 or 35 d).

Canola germination and growth

Canola (*Brassica napus* cv. Westar) seeds were sterilized in 15% bleach for 10 min under constant agitation, then transferred to sterile, moist silica sand covered with Saran Wrap® for 3 d. Seeds of approximately the same size were transferred to soil-filled borosilicate tubes, and treated as described in the wheat germination and growth section. Plants were watered with 15 mL of water every third day for the duration of the experiment (35 d).

Soil phenanthrene dosing

When dose response tests were conducted, concentrations were between 0-1000 mg kg^{-1}. These values were determined from previous literature studies (Salanitro *et al.* 1997; Kelly *et al* 1999; Hou *et al.* 1999) of the toxicity of hydrocarbons to plants. The selected model site also contained approximately 4600 mg kg^{-1} total petroleum hydrocarbons in the C11-C30 range (Envirotest laboratories, Edmonton, Alberta, Canada), so the higher dose concentration of 1000 mg kg^{-1} phenanthrene was approximately one quarter of this value. A higher value of 2000 mg kg^{-1} was originally selected as the highest dose, but preliminary range finding tests showed no significant difference of toxicity between 1000 and 2000 mg kg^{-1}.

Soils were dosed by dissolving appropriate amounts of phenanthrene (Sigma-Aldrich Co.; Oakville, Canada) in 15-25 mL of pentane (Caledon Laboratories, Inc.; Georgetown, Canada). Phenanthrene solutions were added to soils in glass containers and shaken vigorously for 4-5 min. Individual replicates (at least 4 replicates per treatment) were prepared separately. Soils were left in a fume hood overnight and the pentane volatilized, leaving phenanthrene residues in the soil. Soil used in control treatments was treated with 20 mL of pentane without phenanthrene.

2.2.2 Plant Harvest and Isolation of Rhizobacteria

Plastic Kim-Kap™ caps were removed from the growth tubes and drainage stones shaken out after three (wheat) or five (canola, alfalfa) weeks in the growth chamber. Soil cores were then pushed out of their tubes with a wooden dowel from bottom to top. If a soil core could not be pushed out, a sterile stainless steel spatula was used to loosen the soil, facilitating root removal. Care was taken not to damage root systems. Shoots were cut from roots at the base of the hypocotyl (using a clean blade passed through a flame), and placed in labelled, folded paper towels. Roots were then shaken vigorously and tapped against a clean plastic shield to dislodge excess soil. To ensure collection of only rhizosphere soil, all soil aggregates greater than 5 mm in diameter were removed from roots with sterile forceps (modified from Nichols *et al.* 1997 who selected soil aggregates greater than 1 cm). The root system with adhering rhizosphere soil was placed in a Pyrex® dilution bottle containing 90 mL of sterile distilled water and shaken on a mechanical agitator (278 reciprocations min^{-1}; Eberbach Corp., Ann Arbor, USA) with approximately 1 g of 4-mm glass beads to mechanically remove soil from roots. Shoot fresh weights were recorded immediately post-harvest and shoots were placed in an oven. Dry weight measurements were recorded after 72 h at 65°C. Root fresh weights were taken upon their removal from the soil slurries.

Enumeration of heterotrophic bacteria in the rhizosphere

Pyrex® dilution bottles containing root-soil slurries from harvested roots were placed on their sides in a mechanical agitator and shaken on high setting (278 reciprocations min^{-1}; Eberbach Corp.) at room temperature for 1 h. One mL of each slurry was aseptically transferred to a test tube containing 9 mL sterile phosphate buffer (10 mM K_2HPO_4 adjusted to pH of 7.2-7.3 using 1.0 M KH_2PO_4). Mixtures were serially diluted to a final dilution factor of 10^{-6}. Test tubes were mixed by vortex for 10 s at a medium setting between each dilution. One hundred µL of dilutions (10^{-3} - 10^{-6}) were spread plated on each of three Plate Count Agar (PCA; Difco; Sparks, USA) plates that contained 100 mg cycloheximide (Sigma-Aldrich Co.) L^{-1} to prevent fungal growth. Inoculated plates were then placed in a dark cupboard at room temperature (approximately 23-24°C) for 7 d, and isolated bacterial colonies counted. Whenever possible, plate counts between 30-300 colonies were used in calculations. The number of colonies from the three replicate plates were averaged and recorded. The mean and standard error for each treatment was calculated and expressed as colony forming units (cfu) cm^{-2} of root or g^{-1} of soil.

Enumeration of phenanthrene-degrading bacteria in the rhizosphere

Phenanthrene degraders were enumerated using a modified version of the method described by Kiyohara *et al.* (1982). Instead of plating onto an LB plate, 100 µL of 10^{-2} and 10^{-3} dilutions were directly plated on the surface of three plates containing mineral salts medium (MM2). MM2 plates contained 18 mM $(NH_4)_2SO_4$, 1 µM $FeSO_4 \cdot 7H_2O$, 100

μM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$ and 8.5 mM NaCl in 10 mM Na_2HPO_4-KH_2PO_4 buffer, pH 7.0, plus 15 g Bacto Agar (Difco) L^{-1}. After inoculation, plates were sprayed with phenanthrene dissolved in anhydrous ethyl ether (EM Science; Gibbstown, New Jersey), using a JetPak® system (Sprayon Products; Bedford Heights, OH), leaving a white film on the surface of the agar. Inoculated, coated plates were incubated in a dark cupboard at room temperature for 10 d. Phenanthrene degradation initiated by the bacteria was manifested as a clear halo surrounding a colony. Results from the three plates were averaged and recorded as one replicate and expressed as the number of phenanthrene degraders cm^{-2} of root or g^{-1} of soil.

Measurements of root length and surface area

Roots were removed from the distilled water after serial dilution, then washed and refrigerated overnight in 45 mL of clean distilled water. Roots were then placed in a plastic tray containing 30-40 mL of distilled water and placed on an Epson 2000 flatbed scanner. Total root length (cm) and total root surface area (cm^2) were determined from the scanned root images using the WinRhizo program (Régent Instruments Inc.; Quebec City, Canada). Roots were subsequently dried for 72 h at 65°C and weighed to obtain dry weight measurements.

Determination of rhizosphere soil dry weight

To quantify dry weights of rhizosphere soil, dilution bottles containing soil slurries were left overnight to settle. Supernatants were removed using a Pasteur pipette

and remaining sediment was washed through a 2-mm sieve to remove glass beads. Washing solutions were collected in 100 mL polypropylene beakers (Fisher Scientific Co.) and once again left overnight to settle. Beakers were then placed in an oven at 80°C for 24 hours to dry the sediment. Dried soil for each treatment was subsequently weighed.

Statistical analysis

Experiments were analyzed as a one or two factor analysis of variance (ANOVA) using the computer program SigmaStat 1.0 (Jandel Corporation 1993). There were four replicates of each treatment within experiments, and whole experiments were repeated at least twice. When ANOVA indicated that differences observed were more than what would be expected by variation at $\alpha = 0.05$, pairwise multiple comparisons were conducted using Student-Neuman-Keul's test at $\alpha=0.05$.

2.2.3 Analysis of Site Soil

Isolation of bacteria

Sites were difficult to locate due to prior remediation efforts compromising the soil and inaccessibility. A study site was needed that was relatively fresh, accessible, and no significant remediation efforts had been completed. Once a site became available, bags of soil to a depth of approximately 30 cm were collected from three separate locations within the contaminated area, and one location off-site (control) (see section

2.3.1). Soil was placed in heavy-duty plastic bags, wrapped in opaque black plastic and stored at 4°C until required for laboratory studies.

Ten g (fresh weight) of soil was aseptically placed in dilution bottles containing 90 mL of sterile distilled water and 1 g of 4-mm glass beads. Dilution bottles containing the slurries were placed on their sides on a mechanical agitator and shaken for 1 h. One mL aliquots of the mixed slurries were serially diluted into test tubes containing 9 mL of 1 M sterile phosphate buffer to a final dilution of 10^{-6}. Test tubes were mixed by vortex for 10 seconds at a medium setting between each dilution. One hundred µL of dilutions (10^{-3} - 10^{-6}) were spread on three replicates of Plate Count Agar (PCA-Difco) containing 100 mg cycloheximide L^{-1} to prevent fungal growth. Plates were then placed in a dark cupboard at room temperature for 7 d, at which point isolated bacterial colonies were enumerated. Plates containing between 30-300 cfu were used in calculations. Phenanthrene degraders were enumerated using the same method as described in section 2.2.2. Bacterial concentrations were expressed as cfu g^{-1} of fresh soil.

Consortium growth

A bacterial consortium was made by inoculating four separate tubes of trypticase soy broth (TSB; Difco) with one colony of each type of phenanthrene degrader from the selected model contaminated site. Once the consortium was incubated in a shaker for 12 hours (approximately 26-28°C) and tested for phenanthrene-degrading activity, the consortium was centrifuged at 3000 rpm for 5 minutes. The supernatant was aseptically

poured off, and re-suspended in approximately 1 mL of 50% glycerol and 1 mL TSB. This glycerol stock was placed in a -80°C freezer for use in flow-through experiments (see Chapter 3).

Germination of alfalfa and wheat

To compare the ability of potential model plant species to germinate and establish in site soils, alfalfa and wheat seeds were placed in 40 mL of Guy Lake soil. Soils were kept moist by watering every third day, and on day 12 plants were harvested. Roots were extracted from the soil, rinsed, and dried at 65°C for 48 hours. Roots were then weighed and germination efficiency calculated.

2.3 RESULTS

2.3.1 Site Selection and Analysis

A site was selected in Guy Lake, Alberta, northwest of Edmonton, at a Telus Communications, Inc. tower location. The site (Figure 2.1) was contaminated with diesel fuel in spring of 1996 when ammunition punctured the vessel and spilled fuel on surrounding soil (Figure 2.1a). Crews rarely visit the site, so fuel was left to seep into the ground. The site is approximately 8x8 m, sloping slightly down to the northwest, and is surrounded by a plastic-lined gravel berm (Figure 2.1c). Spilled fuel pooled in the northwest corner of the site (Figure 2.1b). The site contains sparse native vegetation, and was hand-fertilized as a remedial action in 2001 and 2002. Total extractable hydrocarbons in the C11-C30 range were 4600 mg kg^{-1} when sampled in 2001 (Enviro-Test Laboratories, Edmonton, Alberta).

Soils were collected from four locations at Guy Lake. Off-site soil was selected as a non-contaminated reference in August 2001. Future visits to the site, however, showed vegetation dieback at the off-site location. Whether this was due to plume migration or drought conditions was not determined, thus "off-site" cannot be used definitively as a descriptor of the treatment. Soils were also collected from a moderately contaminated area in the southeast corner and two samples were taken from the pooled contaminated area.

A

B

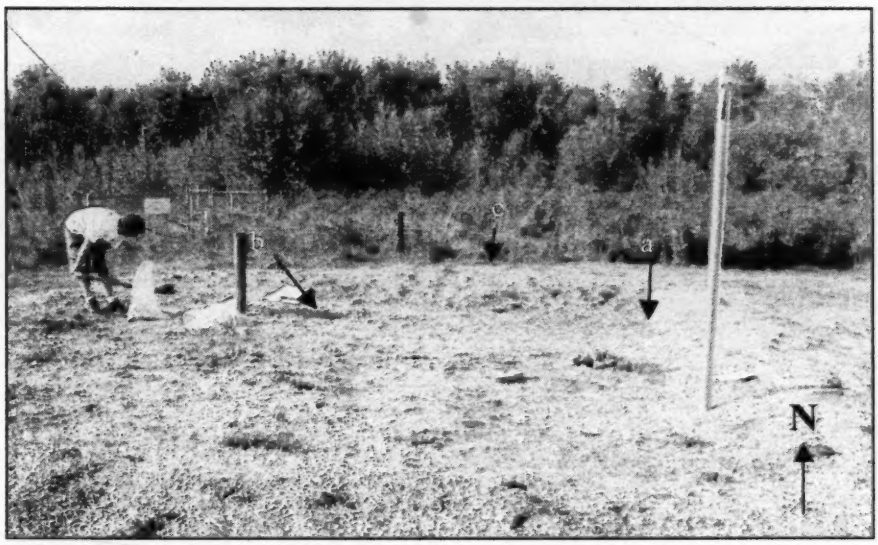

Figure 2.1. Guy Lake, Alberta site in August, 2001 (A) and August, 2002 (B). Diesel fuel leaked out of a holding tank located along the east side of the contaminated area (a) and pooled in the northwest corner of this area (b). A black plastic liner around the exterior, covered with gravel, forms a berm (c) along the perimeter of the spilled area. Red posts are monitoring wells. N indicates north.

Prior to remedial fertilization, enumeration of bacteria from Guy Lake soils showed a significantly higher number of heterotrophic microorganisms in contaminated soil A (Figure 2.2A) compared with the off-site, moderately contaminated and contaminated B soils ($p<0.001$). Conversely, the off-site soils showed the highest number of phenanthrene-degrading microorganisms compared to all locations in the spill area ($p<0.05$; Figure 2.2B).

Selected bacteria were combined to form a model consortium, chosen by their ability to degrade phenanthrene and their location on the site. Microorganisms found off-site were not used in the consortium. Cell morphology observations made using a light microscope revealed the consortium was composed of 9 bacilli. KOH tests were conducted by mixing 3% KOH with an inoculating loop-full of the bacterial colony on a clean slide. A KOH-positive test occurred when DNA from lysed cells formed viscous strings. This positive test suggested a gram negative cell wall structure. Table 2.1 shows the results of KOH tests and colony morphology after 36 h on PCA. All selected bacteria degraded phenanthrene on mineral salts agar sprayed with ethereal phenanthrene.

2.3.2 Selection of Model Plant

Canola was rejected after the first experiment due to high variability, difficulty in germination and susceptibility to aphids. Dose response tests were conducted on *T. aestivum* cv. Maringa was *M. sativa* cv. Beaver.

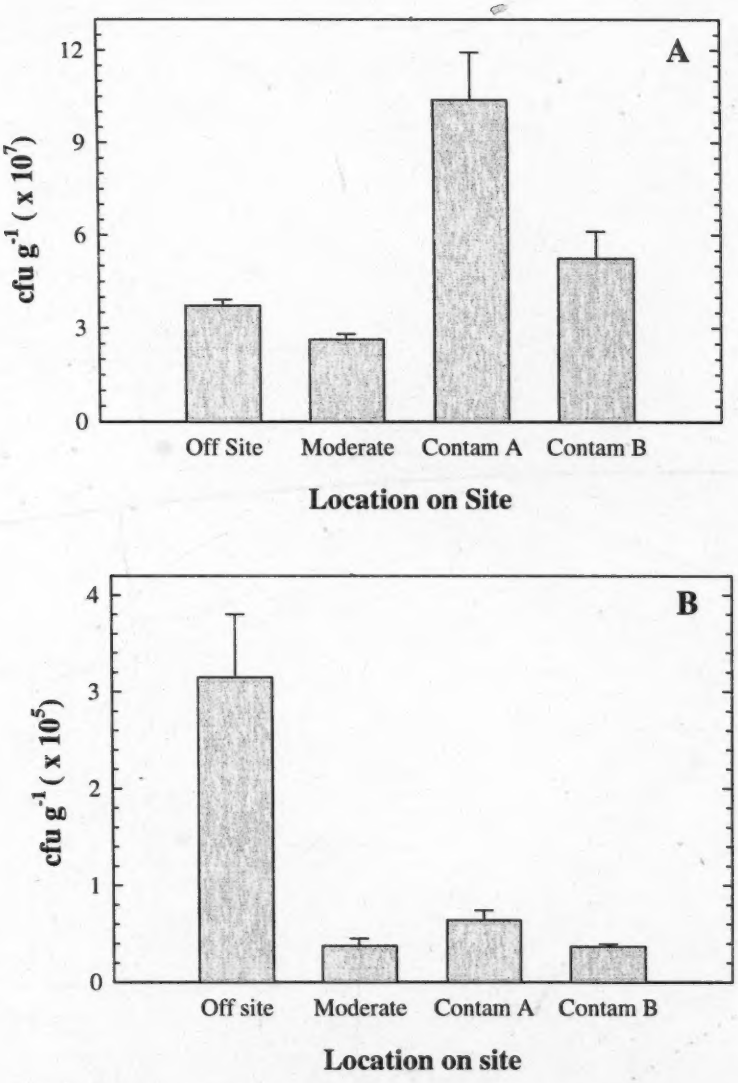

Figure 2.2. Guy Lake soil heterotrophic microorganisms (A) and phenanthrene-degrading microorganisms (B) from soils collected off-site, the moderately contaminated south side (Moderate) and highly contaminated northwest corner of site (Contam A and B). Significant treatment differences exist at $\alpha=0.05$ for both heterotrophic and phenanthrene-degrading microorganisms. Tukey's multiple comparison tests showed heterotrophs were significantly higher in Contam A soil than any other treatment ($p<0.05$), whereas phenanthrene degrader numbers were significantly ($p<0.05$) higher in off-site soil than the other locations within the spill area.

Table 2.1. Colony and cell observations of morphologically distinct model consortium bacteria growing on plate count agar. All bacteria were repeatedly shown to degrade phenanthrene.

Bacterial Designation	Colony Morphology	Colony Colour	KOH Test	Gram Reaction
A	Smooth, Circular, Convex	Dark yellow	-	+
B	Smooth, Circular, Convex	Opaque, Creamy	+	-
Ci	Smooth, Circular, Convex	Opaque, Peach	+	-
Cii	Smooth, Circular, Flat	Clear	+	-
Di	Smooth, Circular, Convex	Opaque yellow	-	+
Dii	Smooth, Circular, Flat	Clear	-	+
E	Smooth, Circular, Convex	Yellow	+	-
F	Smooth, Circular, Convex	Yellow	-	+
G	Smooth, Circular, Convex	Light yellow	-	+

Dose response tests measured parameters including shoot fresh weight, shoot dry weight, root dry weight, root surface area, and total root length. In each dose response experiment, measurements of root surface area and root dry weight were consistent and were used to determine plant response to soil phenanthrene. Phenanthrene was more toxic to wheat roots than to alfalfa (Figure 2.3), however wheat roots were still approximately three times larger and weighed three times more at the higher concentration of 1000 mg kg^{-1}. Statistically, there were no treatment (dose) differences observed with either marker in alfalfa, however phenanthrene was toxic ($p<0.05$) to wheat at concentrations greater than 300 mg kg^{-1} using root dry weight as the marker. Phenanthrene elicited no statistically significant dose response from wheat using surface area as the marker.

Enumeration of phenanthrene-degrading organisms in the rhizosphere demonstrated wheat to contain more cfu cm^{-2} of fresh root at every concentration but the control and the 300 mg kg^{-1} dose (Figure 2.4, Figure 2.5). Both plant species demonstrated a significant ($p<0.001$) increase in rhizosphere phenanthrene degraders in response to increasing concentrations of phenanthrene, however at the highest soil phenanthrene level of 1000 mg kg^{-1}, wheat contained approximately 2.6 times more cfu cm^{-2}. Because the surface area of wheat roots was six times that of alfalfa, in total there were approximately 16 times more phenanthrene degraders in the wheat columns than the alfalfa columns (Table 2.2).

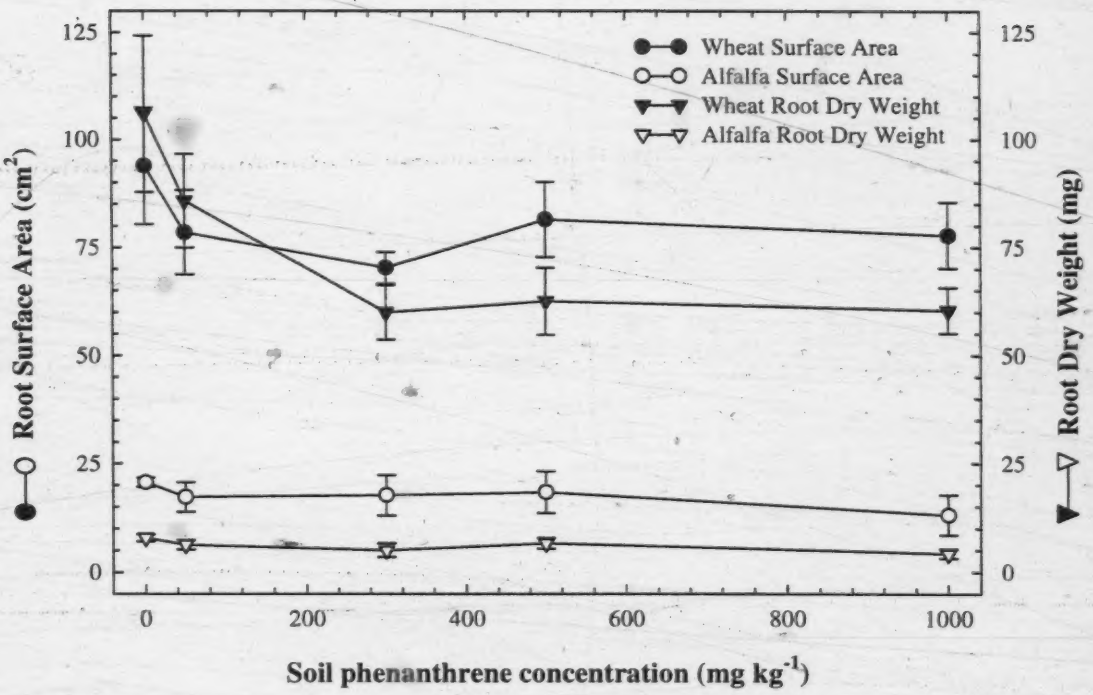

Figure 2.3. Wheat (solid) and alfalfa (open) root responses to soil artificially contaminated with phenanthrene. Root surface area (●—○) was measured using image scanning and WinRHIZO (Régent Instruments Inc.) software, dry weight (▼—▽) was measured after 48 hours at 65°C. A significant treatment effect (α = 0.05) was observed with root dry weight for wheat, but not for alfalfa.

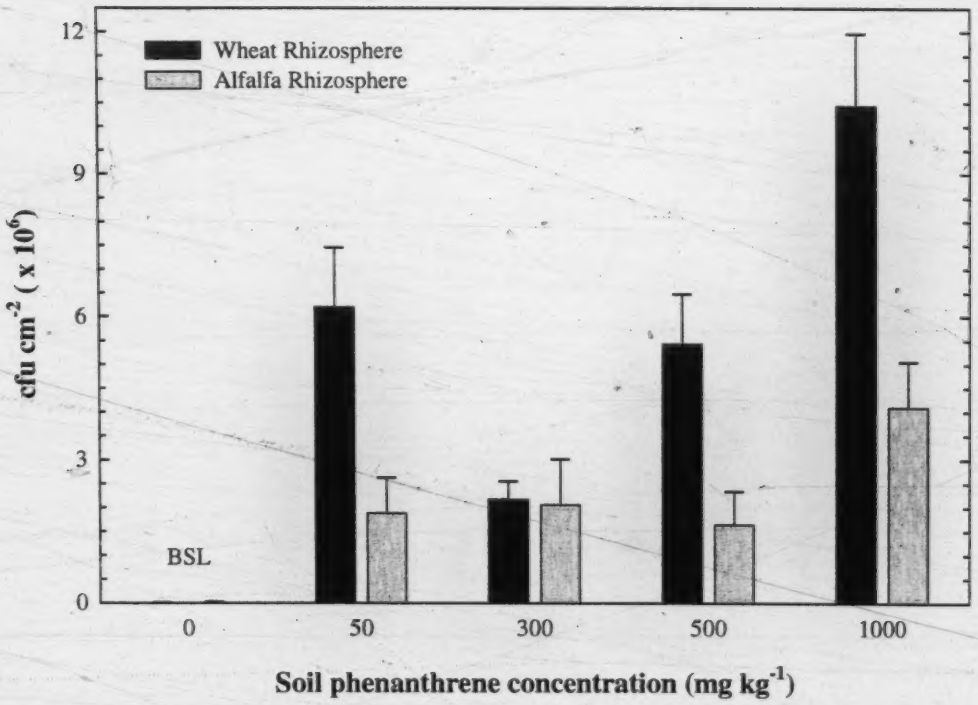

Figure 2.4. Comparison of numbers of phenanthrene-degrading microorganisms in the rhizosphere of alfalfa and wheat. Plants were sown upon radicle emergence and grown 21 d in soil artificially contaminated with phenanthrene. Two-factor ANOVA showed statistically significant differences ($p<0.001$) for soil type and dose. BSL; phenanthrene-degrading microorganisms in soil with 0 mg kg^{-1} were below the scale limit of the graph, although at 3800 and 13 000 cfu cm^{-2} for wheat and alfalfa, respectively.

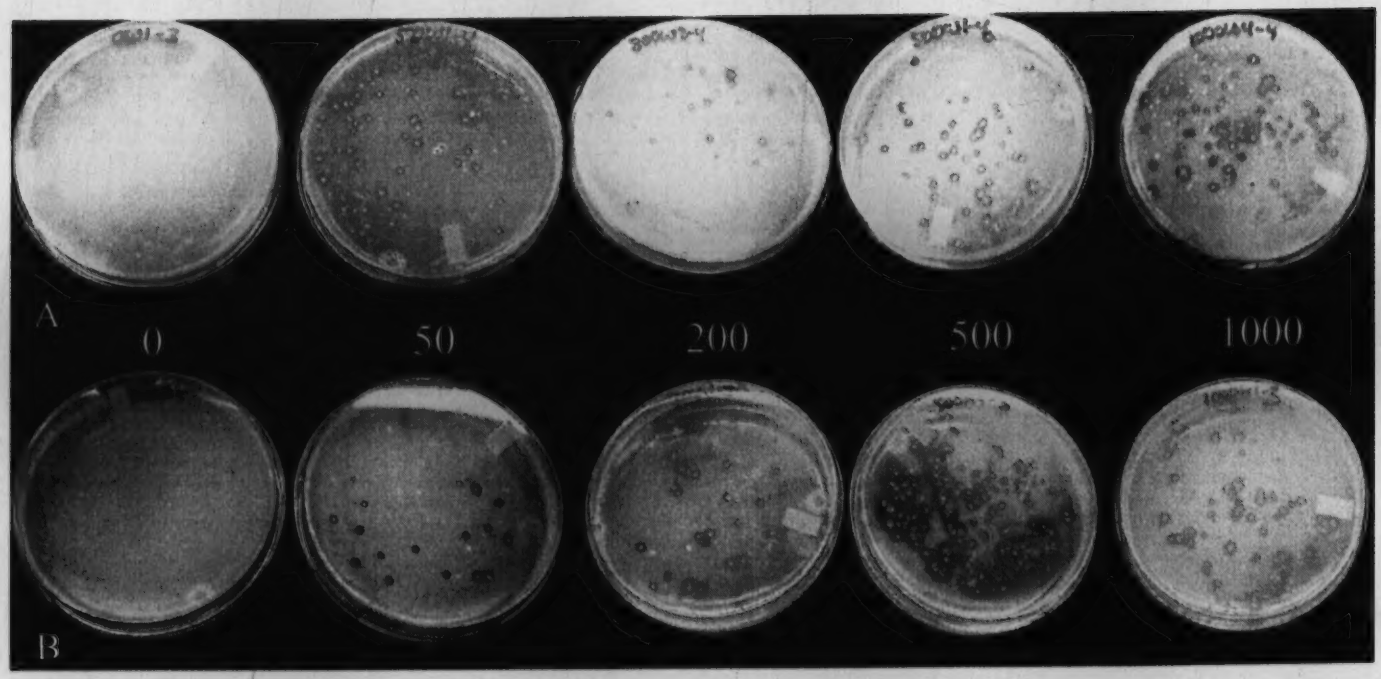

Figure 2.5. Rhizosphere soil phenanthrene-degrading bacteria on mineral salts agar sprayed with ethereal phenanthrene. Colonies surrounded by a clear halo indicate phenanthrene degradation. Wheat dose response at dilution 10^{-4} is shown in plate succession A, B plates represent alfalfa at dilution 10^{-3} . Numbers 0-1000 represent soil phenanthrene concentration in mg kg^{-1}.

Table 2.2. Total number of phenanthrene-degrading bacteria in the rhizosphere of wheat and alfalfa after 21 d in soil contaminated with 1000 mg phenanthrene kg^{-1}.

Plant	Root surface area (cm^2)	Cfu cm^{-2} root	Total rhizosphere bacteria
Alfalfa	13.1	4.09×10^6	4.56×10^7
Wheat	77.8	1.04×10^7	7.81×10^8

Plant species were then tested to determine their ability to germinate in Guy Lake soils contaminated with petroleum compounds. Seed germination efficiency was 100% for both species in a 12-d trial. Plants were harvested and dry weights measured. Although dry weights of wheat roots demonstrated a downward trend in moderately contaminated (35% decrease) and contaminated (50% decrease) soils (Figure 2.6), the mean dry weights were not significantly different at α=0.05. Dry weights of wheat roots were an order of magnitude higher than alfalfa, however the alfalfa roots at that mass (less than 1 mg) were below the analytical threshold of the balance, therefore the actual differences between wheat and alfalfa can not be quantified.

2.3.3 Characterization of the Wheat Rhizosphere

Based on species comparison tests, wheat was selected as the model plant. Further characterization of the number of phenanthrene-degrading bacteria in the rhizosphere was conducted. Two separate comparisons of planted to unplanted columns were conducted. In the first experiment, soils were collected from the *rhizosphere* of columns planted with wheat, whereas in unplanted columns, soils were removed from the upper portions of the soil column (the areas that corresponded to root colonization in planted treatments). When soil collected from the rhizosphere was compared to unplanted soil, main effects due to soil type (rhizosphere, bulk) and dose were significant ($p<0.002$), with no significant interaction. All dose levels, apart from the control, showed

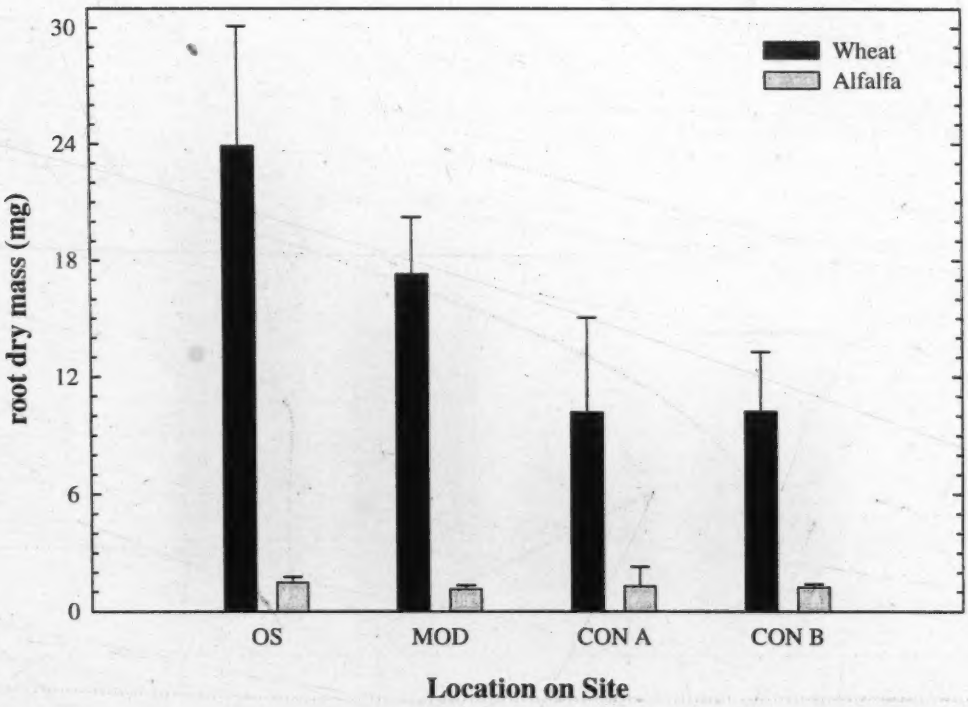

Figure 2.6. Root dry weight of alfalfa and wheat grown in soils contaminated with diesel fuel, collected from Guy Lake, Alberta. Soils were collected to a depth of approximately 30 cm. Off-site soils were labelled OS; moderately contaminated soils, MOD; CON A and CON B soils were both located in the pooled contaminated area, approximately 50 cm apart. Plants were planted from seed and harvested 11 d later. Neither species showed a significant treatment difference.

significantly higher phenanthrene-degrading bacteria in the wheat rhizosphere soil compared to bulk soil (Figure 2.7A). The dose-dependent increase was also significant in unplanted soil columns, however, the magnitude of this difference was 5-15 times lower.

In the second experiment, soils were removed from the *entire* column, without distinguishing rhizosphere from non-rhizosphere soils. When phenanthrene degraders were counted from the entire soil column (Figure 2.7B), fewer differences existed between bulk and planted soil columns. The main effects of soil type and dose were significant, however in this case, there was an interaction noted between soil type and dose ($p=0.005$). *Post hoc* multiple comparison tests showed significant differences between planted and unplanted treatments at 0, 50, and 200 mg phenanthrene kg^{-1} soil. No differences existed between wheat and unplanted soil columns at the higher dose levels of 500 and 1000 mg phenanthrene kg^{-1} soil.

The response of heterotrophic microorganisms to increased soil phenanthrene was also measured from entire soil columns of planted and unplanted treatments. An increase in heterotrophic microorganisms was noticed in both planted and unplanted columns with increasing concentrations of phenanthrene (Figure 2.8). An analysis of variance (ANOVA) indicated a significant main effect of dose, but also showed a significant interaction. The only dose level with statistical difference between whole-column planted and unplanted soils was 200 mg kg^{-1}, with planted columns showing more than twice as many microorganisms as unplanted columns.

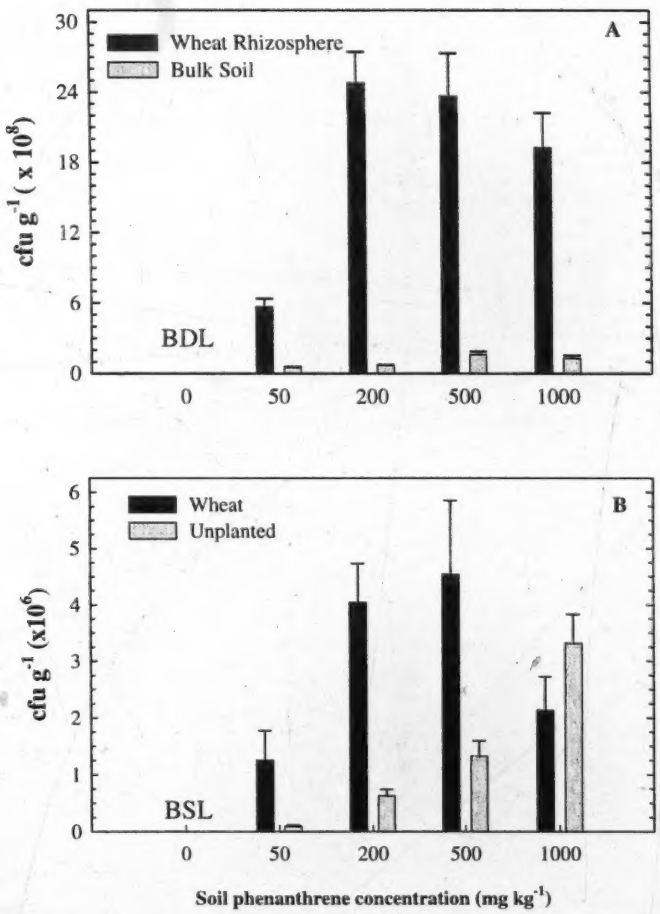

Figure 2.7. Numbers of phenanthrene-degrading microorganisms per gram of dry soil from wheat-planted and unplanted soil columns. **(A)** Wheat soil was collected from rhizosphere only, while bulk soil was collected from the upper portion of unplanted columns. Dose and soil type showed significant treatment differences ($p<0.05$) with no interaction. Plates from the control treatment were below the detection limit (BDL) of 2.9 x 10^7 cfu g^{-1} dry soil **(B)** Measurements from entire soil column; no rhizosphere soil isolation or subsampling. There was a statistically significant interaction between soil type and dose ($p<0.05$). *Post hoc* multiple comparison tests (S-N-K) showed significant ($\alpha=0.05$) difference between planted and unplanted treatments at 0, 50 and 200 mg kg^{-1} soil phenanthrene. Plates from control treatment showed some growth but numbers were below the scale limit of the graph. Wheat columns showed 2800 and unplanted showed 1100 cfu g^{-1} dry soil.

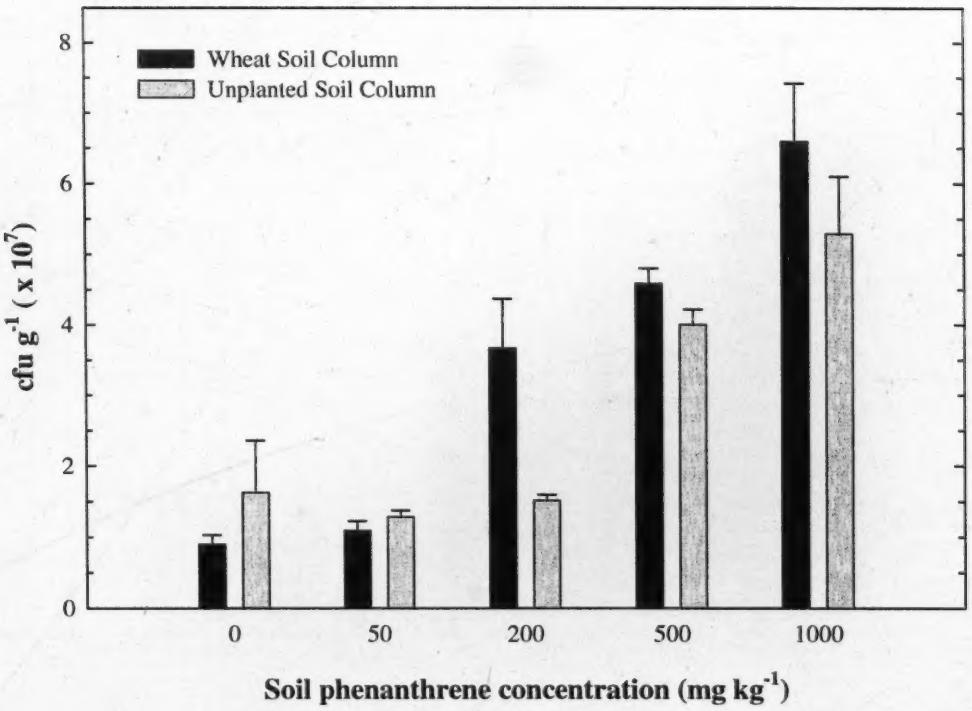

Figure 2.8. Comparison of heterotrophic microorganisms per gram of dry soil from wheat-planted and unplanted soil columns. Measurements were performed on entire soil columns; no rhizosphere soil isolation or subsampling was done in this experiment. There was a statistically significant interaction ($p<0.05$) between soil type and dose. *Post hoc* multiple comparison tests (S-N-K) revealed a significant ($\alpha=0.05$) difference between planted and unplanted treatments at the 200 mg phenanthrene kg^{-1} soil.

2.4 DISCUSSION

2.4.1 Heterotrophic Bacteria in Artificially Contaminated Soil

The presence of heterotrophic bacteria is an important consideration in the decontamination of petroleum-contaminated soil. The ability of some bacteria to break down contaminants as a side effect of metabolic processes could play a major role in the breakdown of PAHs (Cunningham *et al.* 1996; Crowley *et al.* 1997; Siciliano and Germida 1998), though recent studies by Miya and Firestone (2001) suggest cometabolism is not a major contributor to accelerated phenanthrene degradation. Comparisons of planted and unplanted soil columns in this experiment showed an increase in numbers of heterotrophic bacteria in response to soil phenanthrene levels in both planted and unplanted soils (Figure 2.8). There was no significant plant effect (planted vs. unplanted), however there was a significant dose influence and a significant dose-plant interaction. In general, examination of a significant main effect is only appropriate when a significant interaction is orderly (Ott 1993). Data from Figure 2.8 show the order of the means for soil type was not consistent along the dose gradient, so effects based on plant presence or absence may be obscured. The disorderliness of the data can be accounted for by the high standard error at the 0 mg kg^{-1} dose, so it is with caution that a conclusion based on the main effects of plant presence and dose can be made.

In spite of the disorderliness of the main effects data, a significant difference existed between wheat-planted and unplanted columns at 200 mg phenanthrene kg^{-1} (Figure 2.8). If heterotrophic microorganisms are in fact a major contributor to phenanthrene degradation, and heterotrophic numbers indicate increased degradation activity, this finding suggests that planting wheat may be useful at sites moderately contaminated with phenanthrene.

2.4.2 Analysis of Guy Lake Soil

With phenanthrene as the model contaminant, a site contaminated with diesel fuel was not an optimal model site. A site contaminated with PAHs would have been more desirable (for example, a crude oil spill). This would likely have increased the number of bacteria able to degrade aromatic hydrocarbons. Due to availability issues and time constraints, the Guy Lake site was chosen as the contaminated soil. Fortunately, phenanthrene degraders were isolated from Guy Lake soil. Thus, this issue did not pose a problem in this regard.

Unexpectedly, higher numbers of phenanthrene-degrading microorganisms were observed in off-site soils (Figure 2.2B). Although the trend is surprising, it should be noted that the off-site soil supported just over 300 000 cfu g^{-1}, nearly 50 times lower than the number of phenanthrene degraders found in the unplanted treatments of soil artificially contaminated with phenanthrene (Figure 2.7B). So, although the numbers are

higher off-site, they are still quite low in comparison to other PAH-contaminated soils. Keeping the low numbers in mind, these results still seem counterintuitive. The nature of the contamination, however, could offer an explanation. Diesel fuel is composed mainly of long chain alkanes, so there is little selective pressure in a diesel-contaminated soil for microorganisms able to metabolize aromatic ring structures. It is also important to note that the off-site samples were collected from an area supporting vegetation. Although root debris was removed from the soils when tested, it was still likely that these soils had come in contact with live, growing roots. Roots exude a number of compounds containing an aromatic ring, including structures such as morin and biphenyl (Curl and Truelove 1986; Siciliano and Germida 1998). These structural similarities may have influenced the metabolic capacity of rhizosphere microflora, thus explaining the higher numbers seen.

The balanced number of Gram positive and Gram negative isolates in the Guy Lake consortium made it an ideal model consortium to compare the effects of inoculation in planted and unplanted soils. Root-free environments generally provide a more hospitable environment to Gram positive bacteria, while rhizosphere soil is typically inhabited primarily by Gram negative bacteria (Kleeberger *et al.* 1983). The balance of Gram reactions in the selected consortium provided phenanthrene degraders that could be capable of colonizing either of the planted or unplanted treatments.

2.4.3 Selection of Model Plant

Wheat was selected as the model plant due to its favourable response over alfalfa in almost every category (Table 2.3). Phenanthrene was more toxic to wheat, however the higher surface area at higher soil phenanthrene concentrations still made it more desirable as a model species (Figure 2.3). A lethal concentration required to kill 50% of the experimental units (LC_{50}) could not be established, since no tested phenanthrene levels caused mortality. Wheat also successfully established in contaminated soil. The highest phenanthrene concentration tested (1000 mg kg^{-1}) did not even yield an effective concentration to decrease root dry weight by 50% (EC_{50}). Notwithstanding the toxicity of phenanthrene to wheat, it still yielded six times more surface area than alfalfa at high concentrations, a factor important for microbial colonization (Cunningham *et al.* 1996; Alexander 1999). Wheat also supported between two and three times more phenanthrene-degrading bacteria along its surface than alfalfa in less time (Figure 2.4). Dose-response variability was higher in wheat columns, however the difference was negligible and acceptable for the experimental design.

At this stage, it was assumed that if a plant exhibited higher numbers of phenanthrene-degrading bacteria, this translated to increased metabolic capacity in the entire soil column. Using this assumption, the results were consistent with the suggestion by Boyle and Shann (1995) that metabolic activity of rhizosphere organisms is higher in

Table 2.3. Summary of test results with alfalfa and wheat in soils contaminated with hydrocarbons. Overall, wheat demonstrated the more desirable characteristics with shorter experimental time, higher numbers of phenanthrene-degrading bacteria and root surface area. EC_{25} denotes the effective concentration to decrease root surface area by 25%.

Parameter	**Plant Response**		**Favourable Plant**
	Alfalfa	**Wheat**	
Variability	Moderate	High	Alfalfa
Length of Experiment (weeks)	5	3	Wheat
Toxicity of Phenanthrene (mg kg^{-1} EC_{25}; Surface Area as endpoint)	1000	300	Alfalfa
Phenanthrene-degrading bacteria (cfu cm^{-2})*	4.0×10^6	10.5×10^6	Wheat
Germination in Contaminated Guy Lake Soil (%)	100	100	Either
Total root mass (mg)*	4.3	60.4	Wheat
Root surface area (cm^2)*	12.5	77.5	Wheat

*Numbers from 1000 mg phenanthrene kg^{-1} soil

monocots than dicots, although this cannot be concluded, as there was only one representative from each category in these experiments. Furthermore, the methods used in the studies are inconsistent, as all of the experiments in section 2.2 used soil from an actively growing rhizosphere, unlike the experiments of Boyle and Shann (1995) which used soil previously exposed to a plant influence.

2.4.4 Characterization of the Wheat Rhizosphere

Once wheat was selected as the model plant, bacterial populations from the rhizosphere and entire soil column were characterized and compared. A significant rhizosphere effect on the numbers of phenanthrene-degrading bacteria was observed in columns planted with wheat when compared to unplanted columns (Figures 2.7, 2.8). The rhizosphere/soil (R/S) ratio is a comparison of the numbers of bacteria in rhizosphere soil and bulk soil. With respect to heterotrophic bacteria, Cunningham *et al.* (1996) claim that R/S ratios of 5-20 are common. Enumeration of phenanthrene-degrading bacteria from this experiment also falls in that range, with numbers between 5-15.

The dilution of the rhizosphere effect along the dose gradient, in spite of the tightly packed root/soil column, lends some doubt to conclusions obtained by Fang *et al.* (2001) that there is no significant difference between the number of phenanthrene degraders in the rhizosphere and bulk soil in grass species. The major assumption made by these researchers was that in tightly packed soil, all soil is rhizosphere soil. The use of

the term 'rhizosphere' in this case is questionable. If the term 'rhizosphere soil' is substituted by the term 'planted soil' in the conclusions reached by Fang *et al.* (2001), these data do in fact support their findings. At higher concentrations (500 and 1000 mg kg^{-1}), in *planted* soil, there were no significant differences in phenanthrene-degrader numbers, however in *rhizosphere* soil there was a large and significant rhizosphere effect. The discrepancy between these data and those reported by Fang *et al.* (2001) can be explained by the low concentration used by these researchers (5.4 mg phenanthrene kg^{-1} soil).

These data also support the assertion by Ferro *et al.* (1994) that smaller plants exert a more limited rhizosphere effect in the soil, affecting the ability of a plant to accelerate organic degradation. However, some researchers have warned that bacterial numbers are not necessarily correlated to activity (Boyle and Shann 1995; Crowley *et al.* 1997). Before relating these experiments to degradation rates, the *in situ* degradation of phenanthrene in planted and unplanted soils needs to be assessed.

The main objective of this portion of the study was to select a model plant and bacterial consortium for further comparative studies of phenanthrene degradation rates in planted and unplanted soils. The selected plant was wheat and the model consortium was made using nine separate bacterial strains, listed in Table 2.1. These models will be used in a closed soil-plant-microbial system with radiolabelled phenanthrene to assess degradation rates in various systems (Chapter 3).

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CHAPTER 3. USING A MODEL PHYTOREMEDIATION SYSTEM TO TRACE AND MEASURE PHENANTHRENE DEGRADATION

3.1 INTRODUCTION

Using components of the model system described in Chapter 2, a flow-through plant growth chamber was designed with wheat and the model microbial consortium in mind. The objective of this part of the study was to evaluate the effect of wheat on the degradation rates of phenanthrene in autoclaved, indigenous, and inoculated soils.

3.1.1 Phytoremediation

As described in Section 1.2.3, increased contaminant degradation rates in a plant system could be the result of 3 separate mechanisms: 1) plant enzyme mineralization; 2) uptake and sequestration, degradation or volatilization; and 3) plant-enhanced bioremediation. It is widely postulated that rhizosphere-enhanced bioremediation is the primary mechanism of observed plant-mediated increases in phenanthrene degradation rates. Proposed pathways for bacterial degradation are shown in Figure 3.1

The bulk of research into whether phytoremediation is an effective remedial approach for sites contaminated with polycyclic aromatic hydrocarbons (PAHs) has been focussed on the rhizosphere effect. The results of these studies have been widely

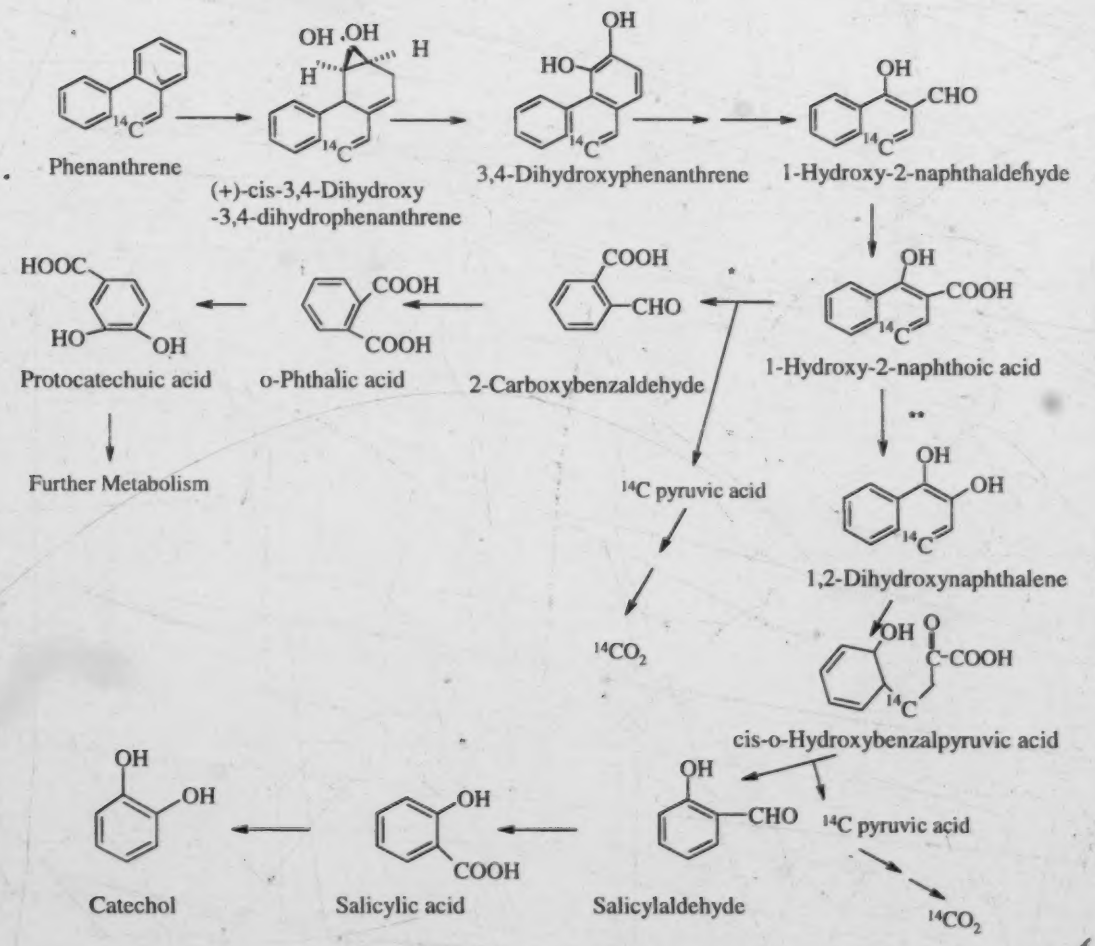

Figure 3.1. Pathways of microbial phenanthrene degradation adapted from Cerniglia (1984). Pathway divergence shows degradation by *Aeromonas* spp. (*) and *Pseudomonas* spp. (**) Radioisotope is located on C9 of phenanthrene (^{14}C)

contradictory. Ferro *et al.* (1994) showed that rates of phenanthrene degradation were the same in planted and unplanted soils, and recorded high rates of phenanthrene degradation (98% disappearance within 96 h). They did not comment on the photosensitivity of the compound and there were no sterile controls to decipher whether the breakdown was a result of microbial mineralization or simply abiotic degradation. Other studies have shown that plant presence has increased phenanthrene degradation rates. After noting enhanced rates of phenanthrene degradation in the rhizosphere of slender oat (*Avena barbata*), Miya and Firestone (2000, 2001) tried to quantify the impacts of individual components of the rhizosphere. Their results suggested that the addition of root exudates and debris to soil accelerated phenanthrene degradation. These conclusions cannot be directly applied to a field situation, since the root exudates used in the soil amendments were collected from healthy, uncontaminated plants growing in hydroponic solutions. These researchers also showed that the impact of the contaminant itself in increasing the number of phenanthrene-degrading bacteria in soil was greater than the exudate effect, and suggested that an increase in cometabolism arising from increased microbial activity in the rhizosphere was not the primary mechanism of the observed acceleration of phenanthrene degradation.

One study has demonstrated surprising rates of degradation and an unexpected fate of radiolabelled phenanthrene. Li *et al.* (2001) tested the effects of a surfactant, TWEEN 80, on the degradation of phenanthrene in laboratory microcosms. These researchers found over 90% of applied ^{14}C-phenanthrene dissipated within 24 d. Seventy

percent of the applied label was found in the shoots of wheat in both control systems and TWEEN 80-treated systems. The unexpected uptake of ^{14}C into shoot tissue could have been due to the open growth chamber design. Soil was applied as a thin layer, left in the light and plants were placed within this chamber. Labelled CO_2 from the photo- and biodegradation of phenanthrene would therefore have been available for assimilation by the plant for photosynthetic processes. This study did not include a sterile or unplanted control, so no conclusions could be reached as to the impact of abiotic, plant or microbial processes on the results. Although the authors could determine there was no ^{14}C-phenanthrene in the shoots, the techniques used did not allow the authors to distinguish between accumulation of $^{14}CO_2$ and ^{14}C-phenanthrene metabolites in shoots. Soil to shoot movement of ^{14}C-phenanthrene would contradict expected transport dynamics (Briggs *et al.*, 1984). Using the log K_{ow} to predict the Transpiration Stream Concentration Factor (TSCF) (see section 1.2.3), phenanthrene is not expected to enter the above ground portion of a plant. However, label may be found in the shoot, as transportation of less lipophilic metabolites into plant tissue may occur (Chaineau *et al.* 1997). Uptake into plant tissues by compounds with a high K_{ow} has been seen by Hulster *et al.* (1994), who found parent dibenzo-*p*-dioxins accumulated in above ground portions of zucchini and pumpkins. With a log K_{ow} of more than 6, uptake of this contaminant was unexpected.

Some studies have found no difference in degradation rates between rhizosphere and bulk soils. Fang *et al.* (2001) used soil collected from a densely rooted pot, assuming

all soil within the pot was rhizosphere soil, and saw no significant difference between phenanthrene degradation rates in planted and unplanted soils. Appropriate controls were used in this case, with a soil-free treatment and a sterilized soil treatment. At a low soil phenanthrene concentration of 5.4 mg kg^{-1}, near complete degradation of phenanthrene occurred over 16 d. These authors also showed that prior exposure of soils to phenanthrene did not improve the capacity to degrade added phenanthrene.

The effect of individual components of the rhizosphere on increased degradation rates are being identified, however extrapolation to field use has been difficult due to experimental design or the complexity of the soil-plant matrix. Some enzymes that have been shown to transform hydrocarbons *in vitro* have been discovered in soils (Dietz and Schnoor 2001), but the relevance of these enzymes *in situ* has not been evaluated. In order to determine the direct result that a plant has on contaminated soil, a sterile control must be used to control for artifacts created by microbial degradation, however this approach has its limitations. Exudate patterns may vary according to the microbial associations in the root zones (Lynch and Whipps 1990; Grayston *et al.* 1996; Prikryl and Vancura 1980). A recent study by Siciliano *et al.* (2003) provided evidence that catabolic potential was greater in rhizosphere soils, in spite of heterotrophic bacterial numbers remaining similar. Bacteria containing genes encoding alkane monooxygenase (*alkB*), naphthalene dioxygenase (*ndoB*), and catechol-1,3-dioxygenase (*xylE*) were isolated from the rhizosphere of Tall Fescue (*Festuca arundinacea*). The presence of this fibrous rooting species increased the degradation rates of total petroleum hydrocarbons (TPH).

In contrast, Rose Clover (*Trifolium hirtum*), a coarse tap root species, did not accelerate degradation of TPH and did not support elevated numbers of bacteria containing *alkB*, *ndoB*, or *xylE*. These data lend additional support to the assertion by Miya and Firestone (2001) that phytoremediation systems selectively enrich soil for contaminant degraders, rather than providing an environment conducive to heterotrophic cometabolic processes.

Uptake of PAHs from soil and subsequent volatilization of metabolites from plant leaves has not, to my knowledge, been demonstrated with petroleum compounds. Cell cultures of *Chenopodium rubrum* have shown degradation pathways of benzo[a]pyrene (Harms *et al.* 1977), however this has not been extrapolated to whole plant degradation.

3.1.2 Flow-Through System

Evaluating the impact of plants on rates of degradation has been attempted, but in most cases, the influence of a live plant on the rates of degradation has been ignored. Sections 2.3 and 2.4 suggested that the numbers of phenanthrene-degrading and heterotrophic microorganisms in the model system used in this study were similar between planted and unplanted soils at the higher concentrations of 500 and 1000 mg phenanthrene kg^{-1} soil. This may suggest that the plant is not likely to influence the rates of phenanthrene degradation. However, Boyle and Shann (1995), Crowley *et al.* (1997), and Siciliano *et al.* (2003) have provided substantial evidence to suggest that bacterial numbers alone are not sufficient to predict the rate of phenanthrene degradation.

To determine the impact of the presence of a wheat plant and a phenanthrene-degrading inoculum on phenanthrene degradation in soil, a system was designed to separate wheat shoots from the soil and root compartment. This system assessed phenanthrene degradation rates under the influence of a live, growing wheat plant and provided an opportunity to determine upward movement of radiolabelled phenanthrene from the soil. Treatments included the presence and absence of a wheat plant in autoclaved, untreated, and inoculated soils. Evolved $^{14}CO_2$ was trapped from root and shoot compartments and radiolabel mass balance was attempted.

The hypothesis was that the presence of a plant and an associated microbial community (indigenous or inoculated) would increase the rate of phenanthrene degradation, and inoculated microorganisms would exert no significant effect in either planted or unplanted soils. The presence of a plant alone was not expected to accelerate degradation rates.

3.2 MATERIALS AND METHODS

3.2.1 Construction of Flow-Through System

Plant chambers

A closed, flow-through plant growth system was constructed. The system (Figure 3.2) is a modified version of the hydroponic high-flow chamber system designed by Orchard *et al.* (2000). Root chambers (30 cm high) were constructed of borosilicate glass with a flat base and a 40/50 mm female ground glass joint at the top. A 10 mm hose connector was located approximately 5 mm from the bottom of the root chamber. Shoot chambers were also 30 cm high, with a rounded top and a 34/45 male ground glass joint at the base. Two 10 mm hose connectors were placed on opposite sides of the shoot chamber, one near the base and one near the top. Root and shoot chambers were separated by a glass blown connector (Figure 3.3A) which was equipped with an inverted glass funnel to guide the plant up to the shoot chamber. The inverted funnel began with an opening of 1.5 cm, tapering to a 5 mm inner diameter (ID) hole at the base. The top of the connector had a male 34/45 mm ground glass joint to connect to the shoot chamber, while the bottom had a male 40/50 mm ground glass joint to connect to the root chamber. Connectors were also equipped with two 10 mm hose connectors; one perpendicular to the connector for a root chamber vacuum hose and another at 45° for watering. A 10 c.c.

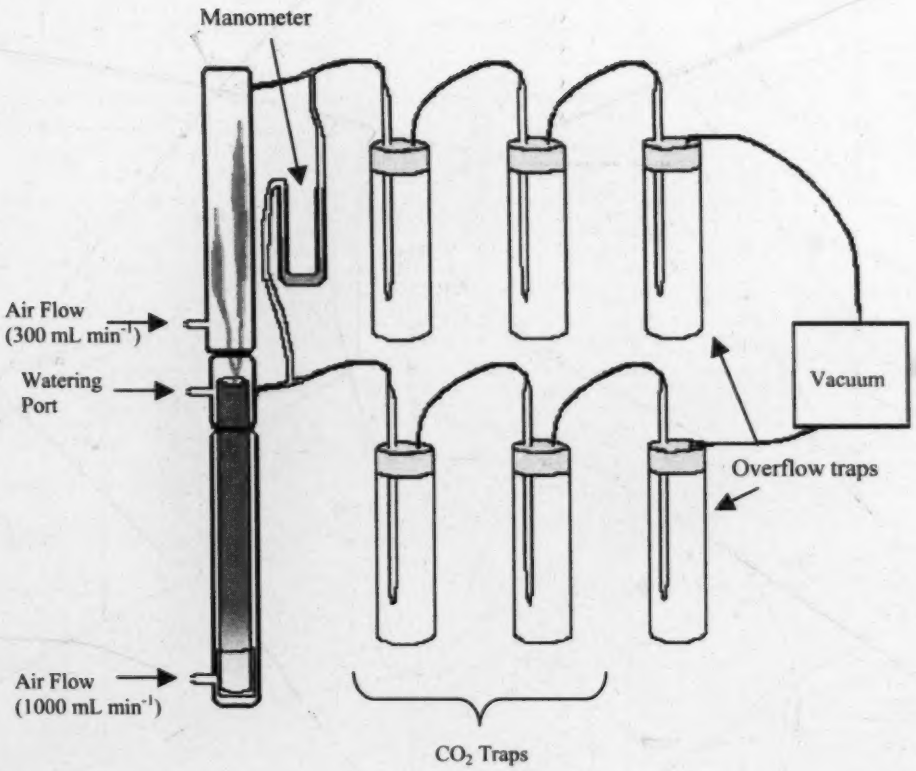

Figure 3.2. Schematic diagram of flow-through system. Air is pulled through ports at the base of both the root and shoot compartments and passed through a series of traps. Flow rates were regulated using a flow meter and hose clamp. The manometer is used to ensure vacuum pressure is higher in the root system in case of seal compromise. There were two CO_2 (KOH) traps and an empty overflow trap as the last in the series of root and shoot traps. Experiments with organic traps used one extra tube in the root and shoot trap area (not shown).

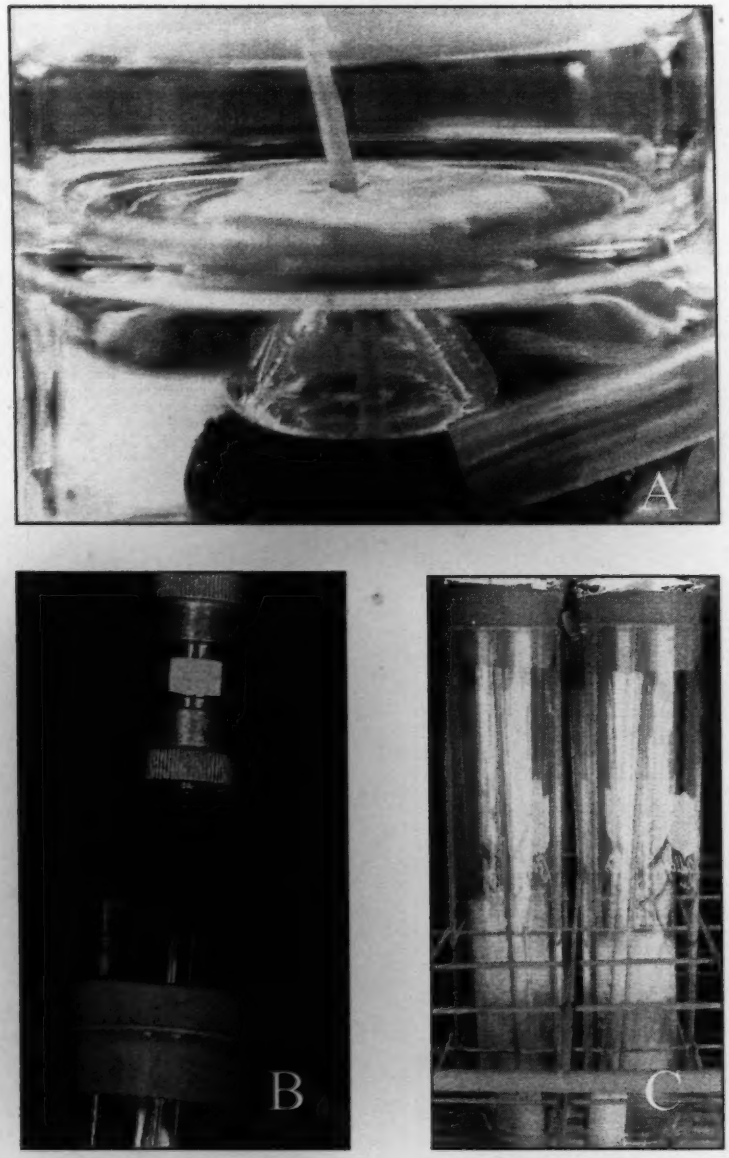

Figure 3.3. Photographs of components of the flow-through system. Connector showing seal, plant, funnel, and watering hose (A); trap stopper design (B), and root traps containing 1.0 M KOH (C).

Luer-lock syringe was fastened to a three-way stopcock and attached via 1/8" ID Tygon® tubing to the root chamber for watering. A plant and soil column, described in section 2.2, could be placed into the root chamber so the surface of the soil met the inverted funnel. The root and shoot chambers were connected to a manometer to maintain higher vacuum pressure in the root chamber, ensuring that no volatile compounds would be mistaken for shoot uptake and evolution if the connector seal was compromised.

Traps

Carbon dioxide and organic traps for the root and shoot chambers were made of 25 X 150 mm borosilicate glass test tubes (Bellco Glass, Inc.; Vineland, NJ, USA). Pyrex® extra-coarse porosity glass gas dispersion tubes (Fisher Scientific Co.; Nepean, Canada) and an 18 gauge needle (Becton-Dickinson Inc., Sparks, USA) were separately inserted through a No. 7 solid neoprene stopper (Fisher Scientific; Figure 3.3B). The dispersion tube was connected to 1/4" ID Tygon® tubing with a stainless steel Swagelok® 3/8 X 3/8" Ultra-Torr vacuum fitting (SS-6-UT-6; Edmonton Valve and Fitting, Inc. Edmonton, AB). The tubing was further connected to 1/8" ID Tygon® tubing with a 4-6 mm reducing connector. Using oil for lubrication, the smaller tubing was threaded onto an Omnifit® ¼-28 thread x male luer-lock adapter (Labcor, Inc.; Anjou, Canada), which was attached to the syringe of the subsequent traps. Each stopper set up was tested for leaks by immersing the stopper and test tube in a bucket of water with an air hose and checking for bubbles. Any leaks were sealed with silicone sealant.

Overflow traps consisted of the same stopper set-up described above, set into 25 X 75 mm glass test tubes. These traps were empty to collect any of the trapping solutions that may have overflowed during the experiment. They were connected to a manifold attached to the house vacuum system and tubing was equipped with a hose clamp for air flow control. Flow meters were attached to the hose connector at the base of each chamber, and flow through the root traps was maintained at approximately 1 L min^{-1}. Shoot flows were maintained at 300 mL min^{-1}. Vacuum pressure was adjusted using a hose clamp downstream of the flow meter. This kept the flow the same but enabled adjustment of pressure.

There were two CO_2 traps per plant growth chamber per root and two per shoot. They were filled with 50 mL of 1.0 M KOH. In replicates with organic traps, the root and shoot compartments were attached to one organic trap each that was filled with 60 mL toluene (Fisher Scientific Canada; Figure 3.3C). Due to difficulties encountered with organic traps in replicate 1 (section 3.4.5), organic traps were not used for testing in blocks 2-4.

Testing CO_2 trapping efficiency of the flow-through system

Seals, trapping, and counting efficiency of $^{14}CO_2$ were tested using radiolabelled sodium bicarbonate ($NaH^{14}CO_3$; New England Nuclear; Boston, USA). Two traps containing 50 mL of 1.0 M KOH were attached to the root and shoot compartments. A glass tube with a Kim-Kap® (Methods, Ch. 2) was placed within the root compartment,

and 0.8 mL (8 888 000 dpm) of radiolabelled $NaH^{14}CO_3$ was placed in the bottom of the column. Nine mL of 1.0 M HCl was dripped from the watering port over the bicarbonate to acidify the solution and release $^{14}CO_2$. Air was drawn through the traps and chambers for 2 h using the house vacuum system. When the vacuum pressure was released, the glass tube was removed and rinsed with 10 mL of 1.0 M KOH. One mL of this rinse was placed into a scintillation vial. Two subsamples of 1 mL from the traps were removed and placed in separate scintillation vials. The volume of liquid remaining in the Kim-Kap™ was also subsampled and 1 mL was placed into a scintillation vial. Ten mL of aqueous counting scintillant (ACS; Amersham Inc. Canada) were added to each scintillation vial. Vials were left to sit overnight in the dark and counted the following day using a Beckman LS 6000 Series Liquid Scintillation Counter with automatic quench correction.

For the first few runs, 2.0 M KOH was used as the trapping solution (Orchard *et al.* 2000), however the ionization of the strong base interfered with the counting process. Glacial acetic acid and 0.5 mL of water was added to the mixture to alleviate the interference of the K^+ and OH^-. This decreased counting efficiency of bicarbonate, likely due to the acidity releasing the bound ^{14}C into the headspace of the vial. The trapping solution was changed to 1.0 M KOH (Chard, Crop Physiology and Water Research Laboratories, Utah State University, personal communication), with no amendments.

3.2.2 Microbial Consortium

Consortium formation

The consortium was formed using the nine phenanthrene-degrading bacterial colonies isolated from Guy Lake soils (Table 2.1). Four replicates (A-D) of 25 mL of TSB were inoculated with one separate loopful of the prepared consortium. Cultures were shaken at 200 rpm on a rotary shaker for 24 h in the dark. To confirm phenanthrene-degrading ability of the consortium, each replicate was plated on two MM2 plates and sprayed with ethereal phenanthrene, then put into glycerol stocks (Chapter 2). Whenever consortia were used in experiments, phenanthrene-degrading ability was checked by spraying an MM2 plate with ethereal phenanthrene.

Growth curve experiments

To determine the growth pattern of the consortia, a growth curve was constructed. Five mL of TSB was aseptically transferred into a sterile 18 X 125 mm test tube. An inoculating loop-full of each of the four consortia (A through D) was placed in a tube of TSB. Tubes were placed in a shaking incubator at 200 rpm at room temperature. Due to heat from the motor, the temperature in the shaker was approximately 26-29°C. Two hundred µL were removed from each tube at time 0 and every hour until 10 h, when samples were removed every 2 h. This continued until the growth rate appeared to begin to level, at which point samples were taken every hour until an asymptotic portion of the curve was reached. Samples were read on a 96-well plate in a spectrophotometer

(μQuant, Bio-tek Instruments, Inc.; Winooski, USA) at a wavelength of 600 nm immediately after removal from the test tube.

3.2.3 Flow-Through System Set-Up

The set-up and analysis steps described below are summarized as a flow chart at the end of this chapter (Figure 3.4).

Soil autoclaving

Soils were autoclaved for 45 min at 121°C, left to sit for 24 h, then autoclaved again for 45 min. Absolute sterility was not achieved in all autoclaved soils. Final sterility of the soil was tested by serially diluting 1 g of the autoclaved soil in 1 M phosphate buffer (pH 7.2) and plating on PCA plates. In most cases, viable, culturable microorganisms were found at very low numbers ($10^0 - 10^3$ cfu g^{-1}) after autoclaving.

Germination of wheat

Wheat seeds were sterilized in mercuric chloride ($HgCl_2$) for 10 min, and then rinsed four times in sterile distilled water before being placed on sterile Murashige and Skoog agar (Gibco BRL, USA) in a magenta vessel. Hypocotyls of seedlings had to be long enough to reach through the inverted funnel of the connector in the flow through chamber. To achieve hypotcotyl elongation prior to the onset of photosynthesis, seedlings were left in a dark cupboard for 4 d.

After 4 d, plant roots were measured in a laminar flow hood on an ethanol-washed acetate sheet with a graph paper underneath. To decrease variation resulting from root system variation, the median root length was calculated and six plants closest to the median value were selected for planted treatments.

Soil dosing and planting

Unlabelled phenanthrene was dissolved in 15-20 mL of pentane. To this solution, 2.5 μCi of [9-^{14}C]-phenanthrene (99% purity; Sigma-Aldrich Co. St. Louis, MO) dissolved in toluene was added to achieve a final phenanthrene concentration of 500 mg phenanthrene kg^{-1} soil. Approximately 600 g of soil and the corresponding phenanthrene solutions were mixed thoroughly in 2 L Wheaton Redi-Pak standard wide-mouth glass jars with TFE-lined lids (Fisher Scientific, Inc.). Dosed soils were left overnight in a fume hood to allow pentane to volatilize.

Soils were poured through a clean, ethanol-washed funnel, into glass columns containing drainage stones and capped with an 18-mm Kim-Kap®. Columns were filled within 6 cm of the top of the tube. The plant was then inserted into the funnel, held gently in place with sterilized forceps, and the remaining soil was poured over the roots. The plant was gently re-positioned after soil pouring. If any plant injury occurred at this point, another plant was used.

To determine the dosing efficiency of each individual treatment, remaining soil was measured into a 22 X 80 mm cellulose extraction thimble (Whatman International, Ltd) and extracted using a Soxhlet extraction apparatus (USEPA SW-486 Method 3540) in methylene chloride (Fisher Scientific Inc.). Extract was evaporated under N_2 and re-dissolved in 5 mL methylene chloride. One mL of this solution was placed in a scintillation vial with 10 mL ACS and counted on a Beckman LS 6000 Series Liquid Scintillation Counter.

Inoculation of columns with consortium

Based on the growth curve experiments described above, consortium B was used for the inoculation of flow through columns. Five mL of sterile TSB were aseptically transferred to a sterile 18 X 125 mm test tube and inoculated with one loop-full of consortium B glycerol stock 11 h prior to planting. Inoculated TSB was incubated at 26-29°C and shaken at 200 rpm. Before inoculation of the soil columns, 200 µL of the consortium was placed in a 96-well plate and the OD_{600nm} was measured in a spectrophotometer to ensure the consortium had reached exponential growth phase. One mL of the incubated consortium was aseptically transferred to 9 mL of phosphate buffer, and then serially diluted in phosphate buffer to 10^{-8}.

One hundred µL of each of the serially diluted tubes were spread-plated on Plate Count Agar (PCA; Difco Inc., Becton, Dickinson and Co., Sparks, MD) for enumeration. Plates were left in a dark cupboard at room temperature for 7 d and plates containing 30-

300 isolated colonies were counted. One mL of the 10^{-4} dilution (achieving a microbial concentration of approximately 1-5 x 10^{8} cfu mL^{-1}) was then dripped into inoculated treatment soil columns. As a solvent control, one mL of sterile phosphate buffer was dripped over soil columns of non-inoculated treatments. The actual concentration of inoculant was determined after plates were counted.

Experimental set-up

Once soils were inoculated and planted, they were watered with 9 mL of sterile, de-ionized water and placed in root chambers. The shoot chambers and connectors were attached. Rope caulk was placed around the hole in the connector in all treatments. Root and shoot air flows were set at 1000 and 300 mL min^{-1}, respectively. The funnel in the connector was placed around the plant and the seal was left open for 3 d, to allow for plants to successfully grow through the hole without injury upon seal closure. Once the plant was growing through the hole, the rope caulk was gently pressed down into the hole with a glass rod, sealing the shoot compartment. Pressures were then adjusted to ensure higher vacuum pressure through the root compartment than the shoot compartment.

The 21-d experiments began upon placement of the columns into the flow-through system chambers. Root and shoot chambers were not separate for the first 3 d of the experiment but higher vacuum pressure in the root ensured no leakage into the shoot traps. If there was leakage, root and shoot evolution fractions would not be separated for the first 3 d. One mL subsamples of root trap solutions were removed and entire trap

contents replaced with clean trapping solutions every 24 h, and shoot traps every 72 h. The subsamples from each trap were placed in a scintillation vial, and the remaining solution poured into a graduated cylinder to measure the exact volume remaining in the tube (some volatilization occurred). Ten mL of ACS was mixed with the subsample and left to equilibrate overnight in the dark. Vials were counted on a Beckman LS 6000 Series Liquid Scintillation Counter, using 1 mL of 1.0 M KOH or toluene (depending on sample) as a background.

3.2.4 Harvest and Soil Analysis

Plant harvest and analysis of tissue

On day 20, 30 mL of 2.0 M KOH was leached through the soil column, to trap any remaining $^{14}CO_2$ as bicarbonate or carbonate. On day 21, subsamples were removed from traps and columns removed from chambers. Shoots from the planted treatments were removed with a clean razor blade and placed in a sterile, pre-weighed test tube. Shoot fresh mass was recorded. Soils were then emptied into pre-weighed 2 L Wheaton Redi-Pak standard wide-mouth glass jars. Fresh soil mass plus KOH was recorded. Roots were removed from soil and excess soil was shaken from them. Shoots and roots were rinsed in 25 mL of de-ionized water, then roots were washed with 25 mL of methylene chloride. One mL from the root rinse was transferred to a scintillation vial. Ten mL of ACS was mixed with the subsample, left in the dark overnight and counted on a Beckman LS 6000 Series Liquid Scintillation Counter.

Rinsed root and shoot tissue were homogenized in methylene chloride using a glass tissue homogenizer. Homogenate was then drawn by vacuum through a 2.1 cm glass microfibre filter (Whatman International Ltd.). Clean methylene chloride was passed through the filter three times as a rinse. The filtrate was evaporated to dryness, then re-dissolved in 2 mL of methylene chloride. One mL was then transferred to a scintillation vial with 10 mL ACS Liquid Scintillation Fluid, left overnight in the dark and counted for ^{14}C. The filter was also removed, placed in a liquid scintillation vial with 10 mL liquid scintillation fluid and counted with the filtered solution.

If radioactivity was found in the shoot filtrate, approximately 100 µL of each sample was also spotted onto a thin layer chromatography (TLC) silica gel plate with fluorescent indicator (60 Å; Whatman, Inc.; Ann Arbor, USA) and developed with a mixture of equal volumes of toluene and ethyl acetate to determine whether the radioactivity was phenanthrene or a metabolite. The TLC plate was placed in an autoradiography cassette with X-OMAT AR film (Kodak, USA), placed in a -80°C freezer for 14 d and radioactive spots on the plate were noted as to whether or not they were phenanthrene by comparing to a stock phenanthrene solution spot on the plate.

Soil analysis for carbon dioxide

Approximately 15 g of soil-KOH mixture was weighed into a 250 mL biometer flask containing 10 mL 1.0 M KOH in the trap arm. Ten mL concentrated HCl was

added to the soil to achieve a pH of less than 2.0, measured using pH indicator paper, to evolve any $^{14}CO_2$ trapped as bicarbonate. Biometer flasks were shaken on a horizontal shaker at 200 rpm for 2 h, then 1 mL of the KOH trapping solution was removed, and placed in scintillation vials with 10 mL ACS. Counts were taken the day following solution transfer and fluid mixing.

Soil analysis for ^{14}C-phenanthrene

Approximately 10 g soil-KOH mixture was weighed into a 22 X 80 mm cellulose extraction thimble. The sample was then ground with a clean mortar and pestle with the same amount of anhydrous sodium sulphate. The mixture was then returned to the cellulose thimble and extracted by Soxhlet extraction (USEPA SW-486 Method 3540). Vacuum grease was used on the ground glass joints to ensure methylene chloride did not evaporate from the Soxhlet apparatus. Samples were dried and re-dissolved in 10 mL methylene chloride. One mL of each sample was transferred to a scintillation vial with 10 mL of ACS, left overnight and counted with a Beckman LS 6000 series Liquid Scintillation Counter. Approximately 100 µL of each sample were also spotted onto a silica gel TLC plate (Whatman, Inc.) and developed with 25% toluene/75% ethyl acetate to ensure phenanthrene was the only compound extracted from column soil. Plates were read under UV light. If any spots appeared that were not phenanthrene, the TLC plate was placed in an autoradiography cassette with X-OMAT AR film (Kodak, USA), placed in a -80°C freezer for 14 d to determine if counted radioactivity was present in any additional compounds.

Soil analysis for ^{14}C metabolites

After Soxhlet extraction, soil from the extraction thimble was poured into a 500 mL Erlenmeyer flask and acidified to pH 2.0 using concentrated HCl and measured with pH indicator paper. Sodium chloride (NaCl) was added in approximately the same volume as the soil, to avoid emulsions. Forty mL of ethyl acetate (Caledon Laboratories, Inc.) were then poured into the flask. Soils were manually, vigorously shaken for 3 min, and ethyl acetate poured off the top of the soil slurries into test tubes that had been heated at 85°C for 24 h. This was repeated 5 times. The total volume of ethyl acetate was recorded and 1 mL of the pooled, mixed extract was transferred to scintillation vials with 10 mL ACS.

Ten µL of each ethyl acetate extract was then spotted on a silica gel TLC plate with fluorescent dye (Whatman, Inc.) and developed in 50% toluene/50% ethyl acetate to determine the presence of metabolites. The TLC plate was placed in an autoradiography cassette with X-OMAT AR film (Kodak, USA) and left in a -80°C freezer for 2 wk to determine the location of radioactivity on the plate. The presence or absence of ^{14}C on the plate was noted but spots were not chemically identified.

3.2.5 Sand Column Harvest

To determine whether imbalances in a mass balance budget could be due to the presence of organic matter in the soil, sand columns were dosed with ^{14}C-phenanthrene in

the same manner as described in section 3.2.3. Three repetitions of unplanted/indigenous sand columns were run concurrently with block 4 of the system flow-through experiments. Analysis of carbon fractions in the sand columns was performed as described in section 3.2.4.

3.2.6 Statistical Analysis

Due to space, labour, and cost constraints, only six treatments could be run during a 3-wk span. As a result, each run was considered a block in a randomized complete block design (RCBD), and four blocks containing each treatment combination were completed. This was a 3 X 2 factorial experiment, with soils as one factor with three levels (autoclaved, indigenous, inoculated) and presence of plant as the second factor (plant, no plant). Both factors are fixed effects. Treatments were randomly assigned to soils and flow-through system chambers using a random number generator (www.randomizer.org). Measured dependent variables included any fraction in which radiolabel was detected, as a percent of the amount applied. Statistics were calculated using a PROC GLM program on SAS. The model used was $Y_{ijk}=\rho_i+\alpha_j+\beta_k+ (\alpha\beta)_{jk} +\varepsilon_{ijk}$ and S-N-K comparisons were done if the ANOVA showed a significant treatment difference. Due to high variability among and within blocks, an alpha level of 0.1 was selected prior to analysis in order to decrease the probability of committing a Type II error.

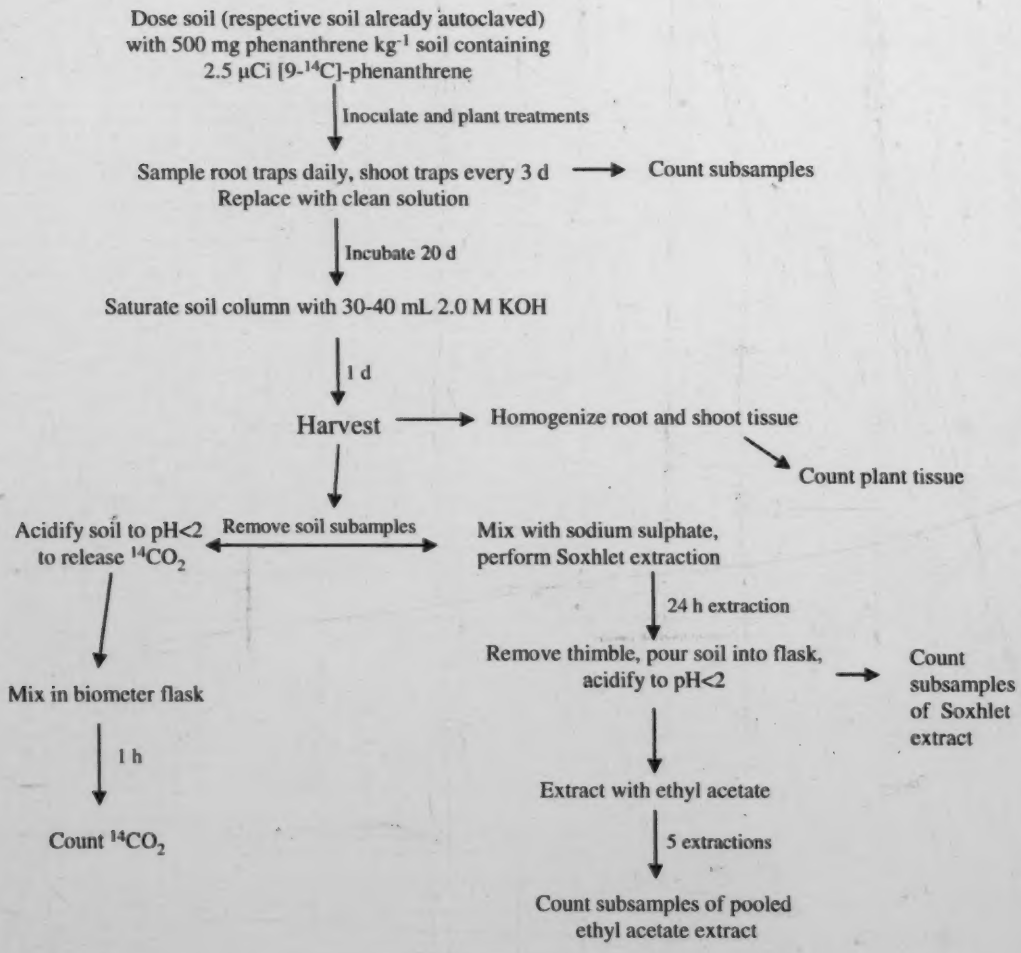
Dose soil (respective soil already autoclaved) with 500 mg phenanthrene kg-1 soil containing 2.5 µCi [9-14C]-phenanthrene
Inoculate and plant treatments
Sample root traps daily, shoot traps every 3 d
Replace with clean solution
Count subsamples
Incubate 20 d
Saturate soil column with 30-40 mL 2.0 M KOH
1 d
Harvest
Homogenize root and shoot tissue
Count plant tissue
Acidify soil to pH<2 to release 14CO2
Remove soil subamples
Mix with sodium sulphate, perform Soxhlet extraction
24 h extraction
Remove thimble, pour soil into flask, acidify to pH<2
Count subsamples of Soxhlet extract
Mix in biometer flask
1 h
Count 14CO2
Extract with ethyl acetate
5 extractions
Count subsamples of pooled ethyl acetate extract

Figure 3.4. Series of steps taken during planting, harvest and soil analysis of flow-through system experiments.

3.3 RESULTS

3.3.1 Trapping Efficiency of CO_2 Traps

Radiolabelled sodium bicarbonate (New England Nuclear) was used to test the trapping efficiency of the carbon dioxide traps. Problems with counting when 2.0 M KOH was used on its own or with acidic amendments, recoveries were between 22-53% (5 separate experiments). When the trapping solution was changed to 1.0 M KOH with no amendments, trapping and counting efficiency increased to 98-100% (three runs).

3.3.2 Growth Curves and Inoculation of Microbial Consortium

Microbial consortium replicates A through D each followed expected bacterial growth patterns. The lag phase was approximately 6 h, the exponential growth phase, approximately 6 h, followed by a stationary phase, at which point sampling was stopped (Figure 3.5). Each curve fit a sigmoidal 3-parameter equation ($y=a/(1+\exp-((x-x_0)/b)$) with r^2 values of greater than 0.99. Replicate B was selected as the stock for flow-through studies based on the time to exponential growth phase (11 h was the most convenient for experimental set-up). For system set up, medium was inoculated with consortium B 11 h prior to soil inoculation to reach the approximate mid-point of exponential growth. Each block of flow-through system replicates was inoculated with 1 mL of a 10^{-4} dilution after 11 h of growth in TSB. Among the four blocks, the inoculum

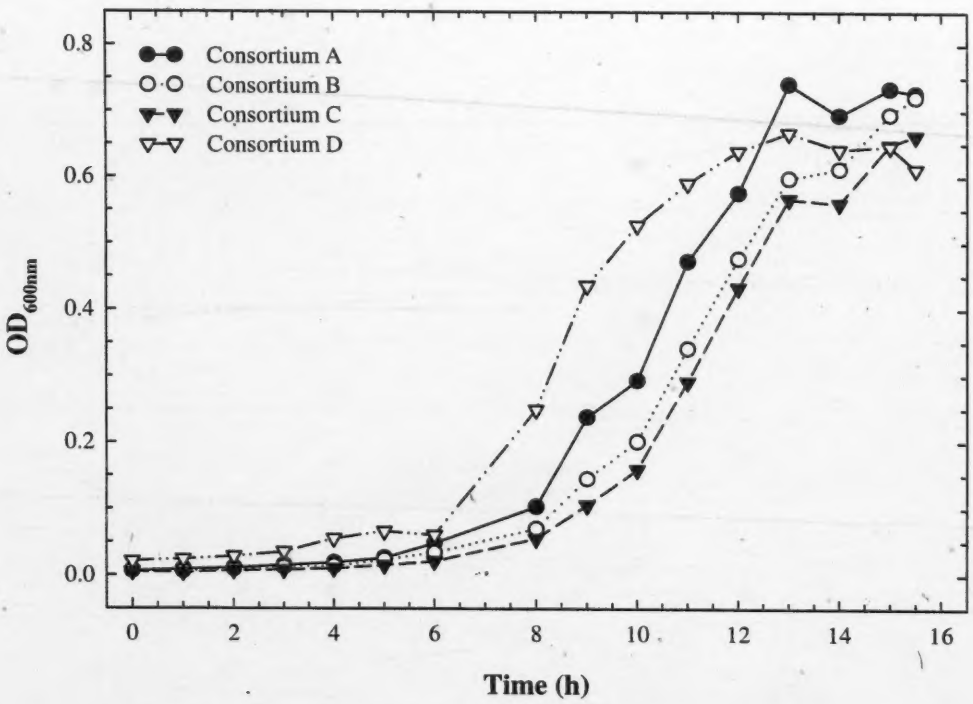

Figure 3.5. Optical density (OD) of phenanthrene-degrading consortia A-D. Replicates were inoculated in TSB and shaken at 200 rpm in a shaking incubator at 28°C. Replicate B was selected as the representative sample for use in flow-through system studies based on the time to exponential growth phase.

density varied between 1.7-16.4 x 10^4 cells per tube, however the measured $OD_{600\ nm}$ was always within the exponential growth phase (Table 3.1, Figure 3.5).

3.3.3 Soil and Plant Harvest

Plant tissue analysis

Among the plants of the four blocks, radioactivity was found in two shoots, at concentrations of less than 0.01% of applied radiolabelled phenanthrene. There was no detectable ^{14}C in any of the other shoots tested. Where radioactivity was found, TLC confirmed it was not phenanthrene, though no further identification was performed.

Analysis of root tissue was extremely difficult, because the addition of the 2.0 M KOH 24 h prior to harvest rendered the roots quite thin, fragile, and difficult to harvest. Roots that were harvestable had rhizosphere soil adhering to the surface, and homogenates contained soil. Radioactivity was detected in root rinses, however due to the contamination of the tissue by rhizosphere soil, it is impossible to determine whether this radioactivity was derived from the root or the adhering soil. Any attempt to manually wash the soil was destructive to the small, fragile root. As a result, root analysis was only performed for block 1. Including adhered soil, the rinsate and root homogenate yielded less than 0.01% of applied radioactive phenanthrene.

Table 3.1. Bacterial counts and OD_{600} of inoculum used in flow-through system experiments.

Block	Stock OD_{600}	Number of bacteria inoculated per tube ($x\ 10^4$)
1	0.516	16.4
2	0.248	3.0
3	0.211	1.7
4	0.447	15.0

Phenanthrene

The amount of phenanthrene detected as recovered ^{14}C-phenanthrene remaining in the columns after 3 wk was significantly affected by planting but not by soil type and there was no significant interaction between the two (α=0.1). Each planted treatment contained between 21-25% of applied phenanthrene remaining, while the unplanted treatment had between 37-46% remaining (Figure 3.6A). The unplanted/indigenous treatment was significantly different from the planted/inoculated treatment only, the unplanted/autoclaved treatment had significantly higher phenanthrene remaining in the column than all planted treatments but was not significantly different from the other unplanted treatments, and the unplanted/inoculated treatment was not significantly different from any of the treatments (Table 3.2.)

Carbon dioxide

No detectable $^{14}CO_2$ was found in any of the shoot traps. Any further mention of $^{14}CO_2$ evolution will refer to root chamber carbon dioxide evolution only.

Analysis of $^{14}CO_2$ evolution showed two outliers. In block 1, 42% of applied phenanthrene was released as $^{14}CO_2$ in the planted/autoclaved treatment compared to 12, 14 and 3 % in blocks 2-4, respectively. In block 2, 39% of applied phenanthrene was released as $^{14}CO_2$ in the unplanted/inoculated treatment, compared to 8, 6, and 8% in

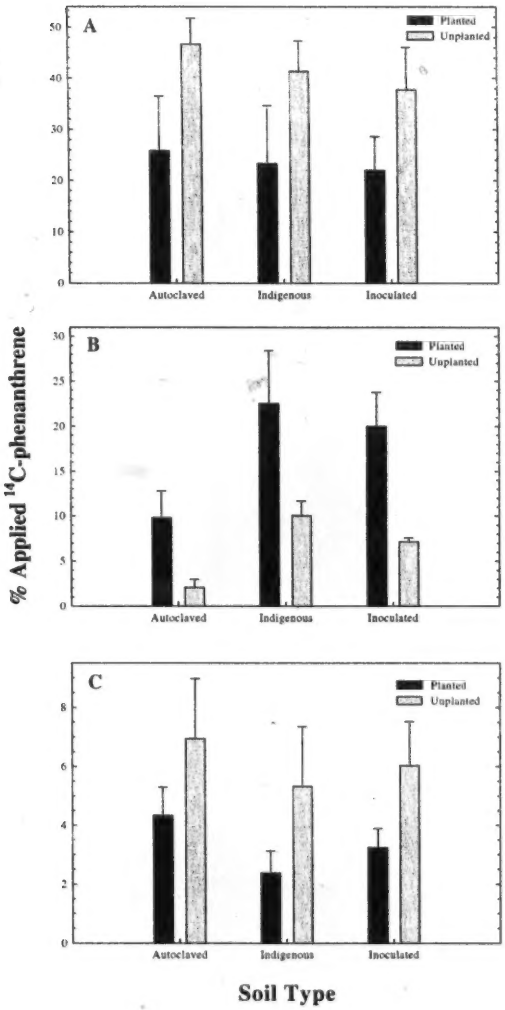

Figure 3.6. Applied radiolabel found in phenanthrene (**A**), CO_2 (**B**), and metabolite (**C**) fractions in planted and unplanted soil columns. **A**. Plant presence or absence affected the remaining phenanthrene significantly, while soil type had no significant effect. **B**. Both soil type and plant presence significantly affected the CO_2 evolution from soil columns. Two outliers were removed from this analysis (see text). **C**. Planted treatments had significantly less label in the metabolite fraction. Soil type had no effect. All significance levels were α=0.1. Treatments were replicated among 4 blocks, and error bars represent standard error.

Table 3.2. Location of ^{14}C in planted and unplanted columns containing [9-^{14}C]-phenanthrene. Means are calculated as a percent of applied phenanthrene. Treatments with different letters are significantly different using LSMEANS/pdiff option in SAS.

^{14}C Fraction	Plant	Soil Type	Mean	
Phenanthrene	No	Indigenous	41	*a b*
	No	Inoculated	37	*a b c*
	No	Autoclaved	46	*a b*
	Yes	Indigenous	23	*a c*
	Yes	Inoculated	21	*c*
	Yes	Autoclaved	25	*a*
CO_2	No	Indigenous	10	*a b*
	No	Inoculated	7.8	*a*
	No	Autoclaved	2.0	*a*
	Yes	Indigenous	22	*b c*
	Yes	Inoculated	20	*b c*
	Yes	Autoclaved	10	*a b*
Metabolites	No	Indigenous	5.3	*a b*
	No	Inoculated	6.0	*a b*
	No	Autoclaved	6.9	*a*
	Yes	Indigenous	2.3	*b*
	Yes	Inoculated	3.2	*b*
	Yes	Autoclaved	4.3	*b*

blocks 1, 3 and 4, respectively. These values were outliers (greater than 3 times the standard deviation when removed from the calculation) and were removed from the analysis. Excluding the outliers, the $^{14}CO_2$ evolved from columns was significantly affected by both soil type and plant presence/absence. There was no significant interaction.

The planted/autoclaved treatment did not evolve significantly more or less $^{14}CO_2$ from any of the unplanted treatments but means were significantly lower than the other planted treatments. The unplanted/autoclaved treatment evolved significantly less $^{14}CO_2$ than the unplanted/indigenous, planted/indigenous, and planted/inoculated soils. The planted/indigenous and planted/inoculated columns evolved significantly more $^{14}CO_2$ than any of the other treatments except each other (Figure 3.6B; Table 3.2).

All treatments showed a lag in cumulative CO_2 evolution of approximately 4 d, except the autoclaved treatments (Figure 3.7). The planted/autoclaved treatment showed a longer lag of 7 d, whereas the unplanted/autoclaved treatment showed minimal activity even at day 20. The rate of degradation between indigenous and inoculated treatments was approximately the same from day 1-day 5, at which point the rate of $^{14}CO_2$ evolution in unplanted treatments slowed. The planted/indigenous and planted/inoculated treatments continued to increase the degradation rate until approximately day 19, when rates decreased (Figure 3.7).

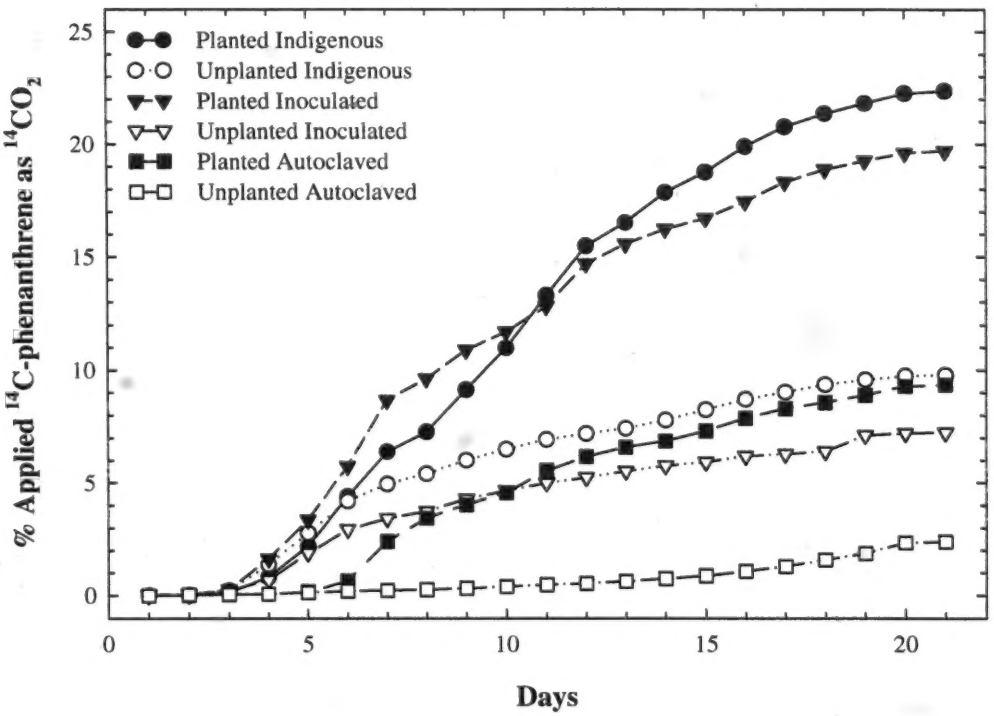

Figure 3.7. Cumulative $^{14}CO_2$ evolution from soils labelled with [9-^{14}C]-phenanthrene as a percentage of initial phenanthrene applied. Dark symbols represent planted treatments, while open symbols are unplanted treatments. Soil treatments included indigenous (circles), inoculated (triangles) and autoclaved (squares). Lines are averages of 4 blocks. ANOVA showed significant main effects of both plant and soil type at α=0.1.

Metabolites

The metabolite fraction was small (between 2-7% of applied radiolabel), however there was a significant plant effect. The amount of metabolite in each fraction was significantly higher in unplanted soil while the unplanted/autoclaved treatment had significantly more metabolites than any of the planted soil type (Figure 3.6C; Table 3.2).

Recovery of ^{14}C

The recovery of ^{14}C was low in most runs. Block 3 showed higher recoveries than the other blocks (average of 77% compared to 57, 55, and 62% in blocks 1, 2, and 4, respectively). It was also the block with the lowest overall phenanthrene degradation.

3.3.4 Sand Column Recovery of ^{14}C

To determine whether the low recovery rates were due to sorption of the parent compound to organic matter, three sand columns were dosed with 500 mg phenanthrene kg^{-1} soil, including 2.5 μCi of ^{14}C-phenanthrene. Daily evolution of $^{14}CO_2$ was measured from these columns, and after 3 weeks, phenanthrene and metabolite fractions were also measured. Evolution of $^{14}CO_2$ was lower in the sand columns, and overall ^{14}C recovery ranged from 59-70%.

3.4 DISCUSSION

Planted soils and associated microorganisms broke down more phenanthrene and evolved significantly higher $^{14}CO_2$ than unplanted soils. This supports the hypothesis that mineralization of phenanthrene in planted systems is higher and phytoremediation does accelerate decontamination of phenanthrene-contaminated soils. These data also support the assertion that the mechanism of degradation is primarily the enrichment of indigenous microorganisms, improving their capacity to degrade the contaminant (Walton and Anderson 1990; Miya and Firestone 2001; Siciliano *et al.* 2003). In addition, these findings support the suggestion that plant exudates may contribute to the overall catabolic capacity of a contaminated soil (Dietz and Schnoor 2001)

3.4.1 Microbial Consortium

The original inoculum density of each block was measured to ensure that any differences seen in inoculated treatments on a per-block basis were not due to differences in the concentration of inoculum. To reach the exponential growth phase and increase the likelihood of being competitive with already established indigenous organisms, cultures were grown for 11 h. Although differences in original bacterial concentrations did exist (Table 3.1), the block variation seen in inoculated treatments did not correlate with original inoculum density.

3.4.2 $^{14}CO_2$ Evolution

These data suggest that inoculation of soil with laboratory-grown phenanthrene-degrading bacteria does not influence the degradation rate of phenanthrene in soil. Although there was a significant soil type main effect when evolution of $^{14}CO_2$ was measured, this difference was observed with the autoclaved soil treatment, whereas the indigenous and inoculated soil phenanthrene degradation rates were not significantly different. Even though there were no statistical differences over the three weeks of this experiment, the lower amounts of CO_2 evolution from inoculated soils (Figure 3.7) suggests the possibility that inoculating soil may in fact decrease or delay soil decontamination over the long term. If degradation rates continued in the pattern suggested by Figure 3.7, management goals may be reached faster in planted, indigenous soils.

3.4.3 Plant Tissue Analysis

As expected, no detectable label was found in above-ground portions of wheat plants in most blocks. Two plants contained less than 0.01% of applied radiolabel in homogenized shoot tissue, and TLC plates confirmed it was not phenanthrene. This label may have come from metabolites entering the root and being translocated to the shoot. These observations are inconsistent with those of Li *et al.* (1999) who found high quantities of label in upper portions of wheat plants. The label detected in shoots by Li *et*

al. (1999) likely originated from $^{14}CO_2$ released from the soil-lava matrix in which the plant was grown, since there was no physical barrier between shoots and the soil.

In this study, root tissues were not analyzed as initially planned due to the size and fragility of the roots, in addition to adhesion of rhizosphere soil to root surfaces. It is likely that there was some sorption to lipophilic root cell surfaces, but the extent of this sorption is likely quite low. Although recovery of ^{14}C was low throughout the experiment, recovery levels were not different between planted and unplanted treatments. This implies that the sorption of phenanthrene to roots is not a major mechanism of available contaminant removal in soils. Furthermore, analysis from block 1 showed a low label recovery (<0.01%) in the root homogenate.

3.4.4 Soil Analysis

The presence of plants significantly increased phenanthrene degradation rates in indigenous and inoculated soils. More polar metabolites were recovered from the unplanted fraction, suggesting that the initial oxygenation by bacteria requiring the enzyme phenanthrene dioxygenase is not the sole rate-limiting factor in the degradation pathway. Proposed bacterial phenanthrene degradation pathways show that C9 (the position of the radio-isotopic carbon) is present in many intermediates, including *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, 3,4-dihydroxyphenanthrene, 1-hydroxy-2-naphthaldehyde, 1-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene, and *cis*-o-

hydroxybenzalpyruvic acid (Figure 3.1). Neither the identity of the metabolites, nor soil enzyme properties were determined, so no conclusions can be drawn regarding where the rate-limiting step occurs along the pathway. A potential explanation for the lower metabolite residence time in planted soils is the possibility that a microorganism able to oxidize phenanthrene would also be able to oxidize an aromatic ring downstream in the pathway. Since planted soils contained less parent compound after incubation, this implies a higher aromatic metabolic activity.

One unexpected finding was the significant difference in ^{14}C fractionation between the planted and unplanted autoclaved treatments. It was hypothesized that the main mechanism of phenanthrene degradation in soils was microbial and the plant would do little but improve conditions for indigenous microbes. However, the significantly higher $^{14}CO_2$ levels evolved from the planted/autoclaved relative to the unplanted/autoclaved treatments suggests a plant effect larger than simply improving the physical properties of the soil-root microenvironment. This could be explained by the autoclaving treatment not sufficiently killing all bacteria. Plate counts conducted on the autoclaved soils prior to dosing show low background bacterial levels (10-1000 cells kg^{-1} soil). These levels did not affect the inability of the unplanted treatment to degrade the hydrocarbon, however the phenanthrene degradation rates increased in the planted/autoclaved treatment. The more robust bacteria that survived the autoclaving may have been enriched along the root surface in the planted treatments and had lower

competition in the less-populated soil, thus increasing phenanthrene degradation. The exudation pattern of plants grown in autoclaved soil may also be different (Lynch and Whipps 1990; Grayston *et al.* 1996) and enzymes exuded from the plant may aid in the cleavage of an aromatic ring. To determine whether the plant effect seen in this experiment is an artifact of the autoclaving treatment, a comparison of sterile sand columns or gamma irradiated columns rather than autoclaved columns should be conducted.

3.4.5 Recovery of ^{14}C

The incomplete recovery of applied ^{14}C posed a problem in the analysis of these results. Because only between 5-60% of the applied ^{14}C was unaccounted for, the absolute degradation of phenanthrene could not be determined. Although comparisons within this experiment are valid, these data do not provide enough information to predict how long it would take a soil under the same conditions to be decontaminated. It was hypothesized that unrecovered label was due to sorption to organic matter, however the sand treatments (lacking organic matter) also showed low recovery of ^{14}C. The loss of carbon was likely in the metabolite fraction. The phenanthrene extraction and KOH trapping were tested for their efficiency, but the ethyl acetate extraction could not be tested quantitatively. Non-radioactive 1,2-hydroxynaphthoic acid was available and used to test the method qualitatively (TLC plates showed recovery of added 1,2-hydroxy-naphthoic acid, but no quantitation was conducted), however no authentic radioactive

metabolites were commercially available. The polar metabolite extraction procedure may have had a low efficiency and could account for some lost ^{14}C. Another potential loss was as volatile organic metabolites. The organic traps in the flow-through experiments were difficult to maintain, primarily due to their volatility and contamination of downstream KOH traps. Additionally, the plants were grown in a communal growth chamber and the odour of toluene was overpowering so after block 1, the organic traps were no longer used in the system. In a similar experiment by Ferro *et al.* (1994), volatile organic traps recovered approximately 0.6% of the applied phenanthrene, so if a loss did occur from the volatile organic fraction, it was probably negligible. An alternative explanation for the imbalance is a lack of homogeneity of the soil mixture. Small subsamples were taken for analysis (10-15% of the column soil) and could therefore have been misrepresentative of the sample as a whole.

3.4.6 General Discussion and Conclusions

These findings suggest that although overall bacterial numbers were not significantly different in wheat-planted and unplanted soil columns (Chapter 2), the increase in rhizosphere bacterial numbers increased the metabolic capacity of planted soil to degrade phenanthrene. This is consistent with reports by Siciliano *et al.* (2003), who showed increased enzymatic activity in the rhizosphere, and research by Boyle and Shann (1995) showing increased mineralization in planted soils. However, it contradicts findings by Ferro *et al.* (1994), Wetzel *et al.* (1997) and Fang *et al.* (2001). The primary

differences in experimental design between this experiment and those with contradictory results are the presence of a live, growing plant (Wetzel *et al.* 1997; Fang *et al.* 2001), incubation of contaminated soil in the dark (Ferro *et al.* 1994), and continuous monitoring of $^{14}CO_2$ over the course of the experiment (Wetzel *et al.* 1997; Fang *et al.* 2001). This experiment also exposed the plants to a higher concentration of phenanthrene in the soil than Fang *et al.* (2001), who only used 5.4 mg phenanthrene kg^{-1} soil.

Ferro *et al.* (1994) found that over 67% of applied radiolabel had evolved as $^{14}CO_2$ and less than 29% remained in the soil (the identity of compounds containing ^{14}C was not determined) after 30 d of treatment. Plants were found to contain approximately 6% of initially applied radiolabel, though the shoots were not separated from the roots in the growth chamber. The incorporation of ^{14}C into plant tissues did not change the rate of degradation between planted and unplanted soils, however the soil phenanthrene concentration was 100 mg kg^{-1}, lower than the concentration used in this study. As described in Chapter 2, rhizosphere microorganisms are not as numerous at 100 mg phenanthrene kg^{-1} as at 500 mg kg^{-1}, which may explain the difference in plant effects between this study and the one conducted by Ferro *et al.* (1994).

The findings in this experiment support the original hypothesis that inoculation of soil columns would not increase overall phenanthrene degradation rates and that planted soils would improve the degradation rates in contaminated soils. One finding that

contradicted the hypothesis was that the presence of a plant alone did accelerate degradation rates, though this may have been an artifact of the autoclaving procedure. To recover more ^{14}C, future studies should focus on improving the metabolite extraction procedure and sampling more of the soil column. Exudation profiles of plants in contaminated and uncontaminated soils, as well as autoclaved and non-autoclaved soils, could also provide valuable information on the mechanisms involved in phytoremediation.

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CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION

The objectives of this research were to develop a model system for laboratory investigation of biological degradation of a petroleum hydrocarbon, assess the extent of hydrocarbon degradation in planted and unplanted soils, and determine the fate of a model radiolabelled contaminant in the model system. These findings are important to understand the mechanisms involved in the phytoremediation of soils contaminated with petroleum hydrocarbons.

Phenanthrene was selected as the model hydrocarbon for degradation studies due to its documented moderate persistence, availability, and toxicity. Wheat was selected as the model plant because the roots provided a higher surface area for microbial colonization and supported a higher number of phenanthrene-degrading microorganisms in rhizosphere soil than alfalfa. A model microbial consortium was obtained using soil contaminated with diesel fuel from Guy Lake, Alberta. There were very low concentrations of phenanthrene-degrading microbiota in this soil, likely due to the type of contamination. Future studies that are looking to find a higher concentration of phenanthrene-degrading microorganisms in contaminated soils should look at soil contaminated by a crude-oil spill.

An increase in phenanthrene-degrading microorganisms was seen in the contaminated wheat rhizosphere compared to bulk soil (Figure 2.7). This rhizosphere effect was expected and is consistent with reports by other researchers (Cunningham *et al.* 1996; Walton and Anderson 1990; Crowley *et al.* 1997), and was significant at soil phenanthrene concentrations higher than 200 mg kg^{-1}. In future studies on the phytoremediation of phenanthrene, a benchmark soil concentration of at least 200 mg kg^{-1} should be used to stimulate the rhizosphere and avoid the risk of concluding that there is no plant effect where one may exist in a field situation. Although there was a significant increase in bacterial numbers in soil adhering to the root surface (the rhizosphere), this effect did not extend to all soil in the planted treatments. The roots exerted no significant effect on the overall numbers in the soil column at higher concentrations of phenanthrene (Figure 2.7). In spite of this rhizosphere effect dilution, the plant-mediated increase in bacterial populations in soil along the root surface was enough to elicit a significant increase in ^{14}C-phenanthrene breakdown (16-18% higher) and complete mineralization (12% higher) at a phenanthrene concentration of 500 mg kg^{-1} soil (Figure 3.6; Figure 3.7).

Results from this study identify inconsistencies in the current literature that need to be addressed to identify the mechanisms involved in phytoremediation. Virtually no radiolabel was found in shoot portions of the plant. This is inconsistent with studies by Li *et al.* (1999), and Ferro *et al.* (1994), who found label from applied ^{14}C-phenanthrene in the shoot of wheat and crested wheatgrass, respectively. The experimental design of

Li *et al.* (1999) made use of an open growth chamber with little air flow within the chamber. These researchers found 70% of applied radiolabel in the shoot. Without a seal separating the upper and lower portions of the plant, Ferro *et al.* (1994) attempted to avoid $^{14}CO_2$ uptake by the shoot by flushing the compartment with air at a rate of 1 L min^{-1}. This brought the total label in shoot tissues to less than 3%. Future studies looking at the fate of a radiolabel should separate the root system from the shoot entirely, to avoid uptake of $^{14}CO_2$ and incorporation of ^{14}C into plant tissues as a result of photosynthetic processes.

Another important factor in phytoremediation studies is the presence of a live, growing plant. The root-soil interface has not been characterized sufficiently to separate components and base conclusions on the assumption that they are functioning in an additive manner. Comparison of these results with other studies (Boyle and Shann 1995; Crowley *et al.* 1997; Miya and Firestone 2001; Fang *et al.* 2001) suggests that the live plant root affects the system by more than the simple exudation of carbon compounds, and removing this influence could affect conclusions regarding plant effect.

An effective phytoremediation program requires accurate predictions of the ability of a plant to accelerate degradation in a contaminated soil. Basing these predictions on the number of bacteria in the soil should be avoided. In these experiments, the numbers of bacteria in the rhizosphere were higher than in unplanted soil, but this effect was diluted in the overall soil column. In spite of this dilution, the plant still

exerted an effect on the ability of the soil organisms to degrade phenanthrene. Nonetheless, it is important to look at both bacterial numbers and bacterial activity in soils. These findings are consistent with reports by Boyle and Shann (mineralization of 2,4-D; 1995) and Siciliano *et al.* (aromatic hydrocarbons; 2003), who showed increased metabolism in planted soils that contained similar numbers of bacteria compared to unplanted soils. Future phytoremediation studies also need to define their 'rhizosphere'. A pot containing densely rooted soil should not be assumed to contain only rhizosphere soil (i.e. Fang *et al.* 2001).

The primary finding in this study is that plants improve the capacity of indigenous bacteria to degrade phenanthrene by 10-15% (Figure 3.6). The results are consistent with the assertion that a metabolic increase in planted soil stems from selective enrichment of phenanthrene degraders in the rhizosphere, rather than providing a more conducive environment for cometabolism (Miya and Firestone 2001; Siciliano *et al.* 2003). These mechanisms are not mutually exclusive, however, and cometabolism may also have been occurring in the rhizosphere.

These findings are important to regulatory agencies and industry members who are looking to decontaminate moderately-contaminated sites. While the planting of contaminated sites is not a short-term clean up option, it could provide a long-term, cost-effective means of accelerating the decontamination process. To fully understand the

mechanisms involved, the individual roles of each component of the system need to be identified while maintaining the integrity of the system.

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